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(54) Title: RECOMBINANT VIRUS (57) Abstract <p>A nucleic acid which encodes a polypeptide which produces a protective immune response against an alpha-virus such as a Venezuelan Equine Encephalitis Virus, in a mammal to which it is administered, said nucleic acid lacking a competent nuclear targeting signal in the capsid gene. The nucleic acids of the invention are expressed at enhanced levels in vectors such as adenovirus vectors. In particular there is described a deletion mutant of an alpha-virus. Vectors such as recombinant adenoviruses which express the deletion mutant are potentially of use as vaccines.</p>		

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Recombinant Virus

The present invention relates to the production of vectors which express alpha-virus genes, such as recombinant viral
5 vectors like adenovirus. The invention further relates to prophylactic and therapeutic vaccines which are protective against these alpha-viruses, such Venezuelan Equine Encephalitis Virus (VEEV), as well as nucleic acids which are used in the vectors, and methods of treatment using the
10 vaccines.

The structural proteins of the alphaviruses are translated from a 26s RNA. The genes encoding these proteins are contained within a single open reading frame in the order:

15

capsid-E3-E2-6K-E1.

The capsid protein is also known as the "core" and both terms are used herein.

20

Each protein is either co- or post-translationally cleaved from the poly-protein precursor.

Many prophylactic and therapeutic vaccines rely on the use
25 of recombinant viruses such as vaccinia virus, or adenovirus including replication competent and replication defective adenovirus, for effective delivery of the immunogens.

However, there are sometimes difficulties associated with
30 the expression of alpha viruses in such vectors.

The applicants have found that deletion of a region of an alphavirus gene improves expression in certain vectors and allows expression in other vectors which could otherwise not
35 be made. This is useful in vaccine production.

Thus according to the present invention there is provided a nucleic acid which encodes a polypeptide which produces a

protective immune response against an alpha-virus in a mammal to which it is administered, said nucleic acid lacking a competent nuclear targeting signal from a capsid gene thereof.

5

The nucleic acid as described above may be expressed at enhanced levels, for example in an adenovirus.

10

As used herein, the term "polypeptide" encompasses short polypeptides as well as proteins. The expression "enhanced" means that the expression level of the polypeptide is increased as compared to that which would occur if an otherwise similar nucleic acid including the said portion of the nuclear targeting signal were present.

15

The nuclear targeting signal (NTS) of any particular alphavirus is either known or it can be determined by alignment with sequences which resemble known nuclear targeting signals in other alphaviruses. For instance, Jakob, Preparative Biochemistry (1995) 25: 99-117 shows the nuclear targeting signal in the Semliki forest virus. An example of a nuclear targeting sequence of VEEV is described below. These sequences are present in the gene encoding the core or capsid protein.

25

In general, the nuclear targeting signal will be located in a lysine rich area of the genome and will comprise a region which has at least 3 and generally 4 adjacent lysines such as described by Chelsky et al., 1989, Mol + Cell Biology, 9, p2487-2492. In the nucleic acids of the invention, the NTS is inactivated either by complete or partial deletion, or by mutation, for example to alter at least some of the lysine residues.

30

The nucleic acid of the invention may be a DNA or an RNA molecule, suitably a cDNA. Furthermore it suitably encodes a polypeptide which comprises at least one structural protein of said alpha-virus, and most preferably all of

these. Thus, in a preferred embodiment, the recombinant nucleic acid of the invention encodes at least the capsid-E3-E2 proteins of the alpha-virus and more preferably the capsid-E3-E2-6K-E1 proteins of an alpha-virus, provided that
5 the region which encodes the capsid protein lacks a competent nuclear targeting domain.

One alphavirus which has been found to benefit particularly from the present invention is a Venezuelan Equine
10 Encephalitis Virus (VEEV). This virus, is a mosquito-borne alphavirus which is an important cause of epidemic disease in humans and of epizootics in horses, donkeys and mules in certain parts of the world, in particular the South Americas.

15 The existing VEE vaccine, TC-83, was initially produced by attenuation of the Trinidad donkey strain (TRD) of VEE by sequential passage in guinea pig heart cell cultures. However, this vaccine is generally regarded as being inadequate for human vaccination. This is mainly due to the
20 high incidence of side effects in vaccinees and the large proportion of vaccinees who fail to develop neutralising antibodies (Monath et al. 1992, Vaccine Research, 1, 55-68).

A vaccinia-based vaccine against VEE has been constructed
25 (Kinney et al. J. Gen. Virol. 1988, 69, 3005-3013). In this recombinant, 26S RNA encoding structural genes of VEE were inserted into the NYCBH strain of vaccinia. The recombinant virus protected against sub-cutaneous challenge but had limited efficacy against aerosol challenge with VEE.

30 Attempts to express the full length sequence of this virus in adenovirus, a particularly useful virus for vaccine production, failed completely. However, certain deletion mutants could be successfully expressed. It has been found
35 that the virus proteins will be expressed from a recombinant adenovirus which lacks at least some, and suitably all of nucleotides 7749-7887 within the VEEV genome.

- All DNA sequence co-ordinants on the VEEV TC-83 strain followed those of the Trinidad Donkey virus strain (R.M. Kinney et al. Virology (1989) 170, 19-30). The cDNA of the 26s mRNA encoding the TC-83 structural region is that
- 5 reported by Kinney et al (1988) supra, the content of which is incorporated herein by reference. The virulent Trinidad donkey strain of VEE and the attenuated strain TC-83 have both been cloned and sequenced and the amino acid and nucleotide numbering system used in this reference will be used
- 10 hereinafter. This work revealed that there are seven amino acid changes between TRD and TC-83. The majority (five) of these changes occur within the gene encoding the glycoprotein E2. The applicants have found a further three changes over and above those described by Kinney as detailed below.
- 15 The deletion or omission of a nuclear targeting domain has also been found to improve the expression of other VEEV encoded proteins in other vectors such as plasmids.
- 20 Deletion of corresponding regions in other alphaviruses should produce similar enhancements in expression.
- The recombinant nucleic acids of the invention may be prepared by any of the well known techniques used in
- 25 recombinant DNA technology. They may be prepared ab initio using the available chemical methods, for example the automated chemical synthesisers. Alternatively, they may be prepared from wild-type alpha viruses, using known recombinant DNA techniques to generate deletion mutants.
- 30 Thus in a further aspect the invention provides a deletion mutant of an alpha-virus, which mutant lacks a nuclear targeting domain, such as a region corresponding to nucleotides 7749-7887 of VEEV. Corresponding regions in
- 35 other alphaviruses could be readily be determined by comparing sequences and determining analogous regions as is understood in the art. Such comparisons can be made by computer programs.

Confirmation of the nature of the nuclear targeting domain can be confirmed, for example using labelled fragments which may or may not include the purported nuclear targeting domain, and examining where the label appears when the fragments are located in cells transfected with the fragments. The labels may be radiolabels or fluorescent labels as are well known in the art.

- 10 Preferably the deletion mutant of the invention is a deletion mutant of VEEV.

A further aspect of the invention provides a recombinant nucleic acid which encodes a deletion mutant of an alpha-virus as described above.

Nucleic acids of the invention are suitably incorporated into vectors, in particular virus vectors like adenovirus (which may be either replication competent or replication defective), vaccinia virus, or in other expression plasmids where they are under the control of a suitable promoter as understood in the art. Preferably the nucleic acids of the invention is incorporated into an adenovirus and most preferably a replication defective adenovirus.

25 These viruses or plasmids can form vaccines. For this purpose, they will suitably be combined with a pharmaceutically acceptable carrier. Virus vectors are preferably combined with a liquid carrier in an injectable formulation. Plasmid vectors may also be made into an injectable formulation or they may alternatively be bound onto a solid carrier such as a gold bead, which are suitable for administration by means of a gene gun, to the skin of a patient.

35 In yet a further aspect, the invention provides a method of producing a protective immune response to an alpha-virus, said method comprising administering to a mammal a vaccine

as described above. The protective immune response may be used both in prophylaxis and in therapy. Suitable doses will be determined by clinicians taking into account the nature of the patient, the nature of the alphavirus and in
5 the case of therapeutic treatment, as well as the precise nature and form of the vaccine. However in general, when using a virus vector, dosages of the vector will be of at least 10^4 pfu. For instance, in the case of vaccinia vectors or replication competent adenovirus, dosages are suitably in the
10 range of from 10^4 - 10^{12} pfu (pfu = particle forming units). Replication defective adenovirus may have to be administered at higher dosages.

The invention will now be particularly described by way of
15 example with reference to the accompanying diagrammatic drawings in which:

Figure 1 is a diagrammatic map (not to scale and not inclusive) showing the structure of VEEV cDNA including
20 restriction sites and the coding regions for the various proteins;

Figure 2 shows the sequences of various linkers and adaptors used in the constructs of the examples; and
25

Figures 3-28 are the cloning diagrams referred to in the Examples.

In the following Examples, the abbreviations used are as
30 follows:

VEEV	Venezuelan Equine Encephalitis Virus
TRD	Trinidad Donkey Virus Strain
C	Core
E1	Structural protein 1
35 E2	Structural protein 2
E3	Structural protein 3
6K	Structural protein 6K
CMV	Cytomegalovirus

	IEP	Immediate Early Promoter
	Term	Terminator signal
	cDNA	Complementary DNA
	RAAd	Recombinant Ela- Adenovirus
5	NCR	Non-Coding Region
	ATG	Start Codon
	MCS	Multiple Cloning Site
	NTS	Nuclear Targeting Signal
	RBS	Ribosome Binding Site
10	nt	Nucleotide
	nts	Nucleotides
	PCR	Polymerase Chain Reaction
	Δ	Minus

The cDNA of the 26s sub-genomic RNA encoding the TC-83 structural region is that used in the work described below and was provided on the plasmid pTC-5a as reported by Kinney et al. (1988). J.G.V. 69: 3005-3114. All VEEV TC-83 cDNA sequence co-ordinates described below followed those Kinney and co-workers (Kinney et al. (1989). Virology 170: 19-33) for the VEEV strain Trinidad donkey (TRD). Nucleotide sequence analysis of the TC-83 VEEV cDNA identified three differences in addition to those described (Kinney et al. (1989). Virology 170: 19-33) within the open reading frames of TC-83 and TRD. These changes are; i) within the 6K gene of TC-83 an addition codon GCG is located between nucleotides (nts) 9989 and 9990, ii) within the E1 gene of TC-83 nt 10353 is T, and ii) in E1 gene of TC-83 nt 18897 is G.

30 Example 1

Preparation of deletion mutants

All constructs were cloned into the expression cassette contained within pMV100 or pEVV101. pMV100 has been described previously (Wilkinson and Akrigg. (1992) N.A.R. 20: 2233-2239, Jacobs et al. (1992) J. Virol 66: 2086-2095). The insertion at the XbaI site in pMV100 of the palindromic adapter L1 (fig 2a) which encodes an EcoRI site on an XbaI

fragment generated pEVV101 (Figure 3). Thus this modification adds an EcoRI site in between the CMV immediate early promoter (IEP) and terminator signal (Term) allowing cDNA to be cloned into the expression cassette (or to be
5 excised from it) on a BamHI, XbaI or EcoRI restriction fragment.

Recombinant Ela- adenovirus 5 (Rad) was generated using materials, including the shuttle vector pMV60, and methods
10 described elsewhere (Wilkinson and Akrigg. (1992) N.A.R. 20: 2233-2239, Jacobs et al. (1992) J. Virol 66: 2086-2095). The VEEV cDNA from pTC-5a was cloned into pEVV101 on an EcoRI fragment generating pEVV102 (Figure 4). The expression cassette containing the VEEV cDNA was subcloned
15 from pEVV102 into pMV60 on a HindIII fragment generating pEVV105. RAD could not be generated with pEVV105 or other plasmids similarly constructed. RAD was made, however, when the VEEV cDNA was cloned in the wrong orientation under the control of the promoter.

20

The truncated 5' non-coding region (NCR) and core gene (5'-C) were deleted from the TC-83 VEEV cDNA to investigate whether RAD could be made containing the E3-E2-6K-E1 and the truncated 3' NCR (E3-E2-6K-E1-3'). pEVV105 was digested
25 with SpeI cutting the VEEV cDNA immediately downstream of nt 8389, three nucleotides into the 5' of the E3 gene. The three deleted nucleotides were restored by the insertion of the palindromic adapter L4A (fig 2b) at the SpeI site generating pEVV110 (Figure 5). L4A also encoded a start
30 codon immediately upstream of the three 5' nts of the E3 gene, which is preceded by a Kozak consensus sequence ACC (Kozak. (1984). Nature 308: 241-246, Kozak. (1986). Cell 44: 283-292, Kozak. (1987). J. Mol. Biol. 196: 947-950) and an EcoRI site. Using the EcoRI site in L4A and that at the 3'
35 of the VEEV cDNA, the cDNA VEEV construct E3-E2-6K-E1-3' was removed from pEVV110 and cloned into pEVV101 generating pEVV108 (Figure 6). The expression cassette containing the VEEV cDNA was subcloned from pEVV108 to pMV60 on a HindIII

fragment generating pEVV109. RAd was generated using pEVV109.

An attempt was made to generate RAd expressing the truncated
5' NCR and core (5'-C) . To clone 5'-C, the plasmid pUC19
(provided by Boehringer Mannheim UK) was modified by
reversing the order of the XbaI and AccI sites within the
multiple cloning site (MCS). A BamHI and SphI adapter
encoding an AccI and XbaI site (L7L8, fig 2c) was inserted
into the MCS of pUC19 to generate pEVV118 (Figure 7). The
adapter L5L6 (fig 2d) provided the 3' 26 nucleotides of
core gene from the AccI site (nt 8361) to the 3' of core at
nt 8386 and an appropriate stop codon on an AccI/XbaI
fragment and was cloned into pEVV118 generating pEVV119
(Figure 8). The 5' NCR and remaining 5' of the core gene,
up to and including nt8360, was removed from pEVV102 on a
BamHI-AccI fragment and cloned into pEVV119 completing the
constructing of 5'-C and generating pEVV120 (Figure 9). The
5'-C fragment was removed from pEVV120 on an XbaI fragment
and cloned into pMV100 generating pEVV121 (Figure 10). The
expression cassette containing 5'-C was subcloned from
pEVV121 into pMV60 on a HindIII fragment generating pEVV123.
RAd could not be generated using pEVV123.

In an attempt to identify the domain responsible for the
failure to generate RAd containing core, the VEEV core gene
was cloned in two halves. The adapter L10L13 (fig 2e) was
inserted at the NsiI site in core between nucleotides 8016
and 8017. This placed an in frame stop codon downstream
of the NsiI site followed by an XbaI site, an in frame start
codon and a second NsiI site. The resultant plasmid
(pEVV129, Figure 11) was digested with XbaI generating two
fragments containing C sequence. One containing the 5' NCR
and the 5' of core up to and including nt 8016 which
immediately precedes an inframe stop codon. The second
containing the 3' of the core gene from nt 8017 which is
immediately preceded by an in frame start codon, up to and
including the stop codon inserted in the construction of

5'-C (i.e. pEVV121). Each fragment was cloned into pMV100 on an XbaI fragment generating pEVV125 (Figure 12) which encodes the 5' of core and pEVV126 (Figure 12) which encodes the 3' of core. The expression cassette were subcloned from
5 pEVV125 and pEVV126 into pMV60 on a HindIII fragment generating pEVV127 and pEVV128 respectively. RAd could not be generated using pEVV127 but was generated from the pEVV128.

10 Three domains within the 5' of core encoded by pEVV125 that might be responsible for the failure to generate RAd virus containing core were identified. These domains are i) the 17 nts upstream of the start codon at nt7562 which form the truncated 5' NCR; ii) the putative ribosome binding site
15 (RBS) between nt 7886-7930 identified by sequence alignment with the alphavirus Sindbis (Wengler et al. (1992). *Virology* 191: 880-888, Geigenmuller-Gnirke et al. (1993). *J Virol* 67: 1620-1626, Owen and Kuhn. (1996). *J. Virol* 70: 2757-2763); iii) a putative nuclear targeting signal (NTS) between nt
20 7751-7885 identified by sequence alignment with the alphavirus Semliki forest virus (Jakob. (1995). *Preparative Biochemistry* 25: 99-117).

To remove the 5' NCR from core, a PCR fragment was generated
25 with primer P2 (fig 2h) and P3 (fig 2i) using pEVV123 as the template. From 5' to 3', P2 contains a GC rich tail, BamHI site, and the 17 nts homologous to the positive strand of the 5' of the core gene from the start codon at nt 7562. From 5' to 3', P3 contains a GC rich tail and BamHI site
30 followed by 14 nucleotides complementary the positive strand the core gene between nt 7726-7739 including a Bsu 36I site located between nt 7733-7739. The resultant PCR product therefore contained BamHI sites which flanked sequence containing the 5' of core from the start codon at nt 7562 up
35 to and including the internal Bsu 36I site (nt 7739). This PCR product was digested with BamHI and cloned into pUC18 (provided by Boehringer Mannheim UK). The BamHI fragment was then subcloned into pUC19 generating pEVV135 (Figure

13). Reconstruction of core gene was completed by inserting the remaining 3' of core from the Bsu 36I site (nt 7735) up to and including the stop codon following nt8386 from pEVV121 on a Bsu 36I - HindIII fragment into pEVV135
5 generating pEVV136 (Figure 14). The core gene was removed on a BamHI fragment from pEVV136 and cloned into pMV100 generating pEVV133 (Figure 15). The expression cassette containing the Core was subcloned from pEVV133 into pMV60 on a HindIII fragment generating pEVV134. RAd could not be
10 generated using pEVV134

To delete the RBS from core, a TfiI site within core was used which necessitated the removal of the TfiI sites at nt641 and 781 in pUC19. pUC19 was digested with TfiI and
15 the sticky ends generated were modified with Klenow before the plasmid was re-ligated generating pEVV145. Core was cloned into the MCS of pEVV145 on an XbaI fragment from pEVV121 generating pEVV146 (Figure 16). To delete either the NTS or RBS from core it was necessary to remove both
20 signals on a Bsu 36I -TfiI fragment (nts 7735-7928) and then restore either the NTS or RBS separately. This was achieved by digesting pEVV146 with Bsu 36I and TfiI and replacing the RBS-NTS fragment with either a Bsu 36I-TfiI PCR fragment encoding the NTS or a Bsu 36I-TfiI adapter encoding the RBS.

25 A Bsu 36I-TfiI PCR fragment containing the NTS was generated using primers L20 and L21 (fig 1j and 1k). L20 is homologous to the positive strand of core gene and encodes a Bsu 36I site followed by the 10 nt from 7735 to 7749, L21
30 encoded 25nts complementary to the positive strand of the core gene between nts7872-7897 followed by a TfiI site. The PCR fragment was cloned into pTA_g (R & D Systems Europe LTD) using the nontemplated-dependent single A added to the 3' ends of the PCR product. The Bsu 36I-TfiI fragment
35 containing the NTS was then excised from pTA_g and cloned into pEVV146 replacing the Bsu 36I-TfiI fragment containing the NTS and RBS and generating core minus (Δ RBS

(CARBS) (pEVV148 (Figure 17)). CARBS was removed from pEVV148 and cloned into pMV100 on XbaI fragment generating pEVV149 (Figure 18). The expression cassette containing Core Δ RBS was subcloned from pEVV149 into pMV60 on a HindIII
5 fragment generating pEVV150. RAD could not be generated using pEVV150.

Core ΔNTS was generated by cloning the adapter L28L29 (fig 2f) into Bsu 36I-TfiI digested pEVV146 generating pEVV151
10 (Figure 19). L28L29 contains nts7735 to 7750 which are adjacent to and continuous with the 42 from nt 7886 to 7921. The NTS is located between nt 7751 and 7885 therefore L28L29 encodes the region of the core gene between the Bsu 36I site and the NTS and the NTS and the TfiI site thus containing no
15 sequence from the NTS and generating CΔNTS. CΔNTS was cloned into pMV100 from pEVV151 on an XbaI fragment generating pEVV152 (Figure 20). The expression cassette containing the CΔNTS was subcloned from pEVV152 into pMV60 on a HindIII fragment generating pEVV153. RAD was generated
20 using pEVV153.

To confirm that the NTS encoded by core was responsible for the failure to generate recombinant adenovirus containing the VEEV core, CΔNTS was restored to the construct E3-E2-6K-E1-3' using adapter L30L31 (fig 2g). Adapter L30L31 contains
25 the 34nt downstream of the AccI site at nt8361 in core to nt 8394 of the SpeI site located in E1 and therefore re-joins core to E3. CΔNTS was removed from pEVV152 on an XbaI fragment replacing 3'-C in pEVV120 generating pEVV159
30 (Figure 21). L30L31 on a AccI-HindIII fragment was cloned into pEVV159 between the AccI site at the 3' of core and the HindIII site within the MCS of pEVV120 generating pEVV160 (Figure 22). CΔNTS +L30L31 was removed from pEVV160 on XbaI-SpeI fragment and cloned into pEVV108 placing core-
35 L30L31 upstream of E3 and restoring the Core-E3 junction generating pEVV161 (Figure 23). The VEEV cDNA CΔNTS-E3-E2-

6K-E1-3' was removed from pEVV160 on an EcoRI fragment and cloned into pEVV101 generating pEVV163 (Figure 24). The expression cassette containing CANTS-E3-E2-6K-E1-3' was subcloned from pEVV163 into pMV60 on a HindIII fragment
5 generating pEVV162. RAD was generated using pEVV162.

Example 2

Confirmation of Nuclear targeting domain

To determine the potential of the putative NTS to locate the
10 Core protein to the cell nucleus, core was expressed as a fusion protein with Green Fluorescent Protein (GFP). The start codon (ATG) was removed from core by replacing the 5' of core in pEVV120 from the BamHI site immediately upstream of the start codon to the unique Bsu361 site within the core
15 gene with a PCR fragment containing an identical fragment of core with the exception that the start codon had been removed. This PCR fragment was generated with the Primers P3 and P15. Within the primer P15 immediately upstream of the BamHI site and downstream of the first nucleotide of
20 Core and additional two nucleotides were included (AT) in order to shift the reading frame two places to the right. This cloning generated the plasmid pEVV188 (Figure 25).

Core minus its start codon was removed from pEVV188 on a
25 BamHI - XbaI fragment and cloned into pUC19 generating pEVV187 (Figure 26). This cloning placed a SalI site downstream of the XbaI site allowing core minus its start codon to be removed from pEVV187 on a BamHI-SalI fragment and cloned into the commercially available vector pEGFP-C2
30 (Clontech, Palo Alto CA. USA) generating pEVV186 (Figure 27). This cloning placed core minus its start codon downstream of and in frame with the GFP gene so that core is expressed as a C-terminal fusion with GFP.

35 When pEVV186 transfected cells were examined by immunofluorescence GFP was found to locate to the nucleus of the cell.

That targeting of GFP-core fusion protein to the nucleus was directed by the NTS encoded by core was demonstrated by generating pEVV172. pEVV172 (Figure 28) was generated by cloning the NTS removed from pTAg (Figure 17) on an EcoRI and SalI fragment and cloned into pEGFP-C2. The NTS was therefore expressed as a C-terminal fusion with GFP. A control plasmid pEVV173 was generated by removing the NTS on a BamHI and BglII fragment and cloning it back into pEGFP-C2 in the antisense orientation behind GFP. In cells transfected with pEVV173, GFP was located throughout the cell and particularly in the cytoplasm. In cells transfected with pEVV172, GFP was located to the nucleus.

Example 3

Immunisation studies

Groups of 10 balb/c mice were immunised with plasmids using the Helios Gene Gun (Bio-Rad, Hercules, CA. USA). The plasmid used were pEVV100, pEVV102 and pEVV163. Each mouse received approximately 5µg plasmid at day 0 and again after 11 weeks. Mice were then challenged 22 weeks following the original immunisation, with 30LD₅₀ of the virulent strain of VEEV TRD subcutaneously. The results are tabulated in table 1. This data suggests that the immune response elicited following immunisation with a plasmid pEVV163 elicited protection while pEVV102 did not. The protective effect seen appears not to be solely the result of an antibody response.

Table 1.

Plasmid	<u>Number of mice surviving 20 days post challenge</u>	<u>Total number of mice</u>
pEVV100	0/10	
pEVV102	0/10	
pEVV163	3/10	

Claims

- 5 1. A nucleic acid which encodes a polypeptide which produces a protective immune response against an alpha-virus in a mammal to which it is administered, said nucleic acid lacking a competent nuclear targeting signal in the capsid gene.
- 10 2. A nucleic acid according to claim 1 which can be expressed in an adenovirus vector.
- 15 3. A nucleic acid according to claim 1 or claim 2 which encodes at least the capsid-E3-E2 proteins of an alphavirus, provided that the region which encodes the capsid protein lacks a nuclear targeting signal.
- 20 4. A nucleic acid according to claim 3 which encodes the capsid-E3-E2-6K-E1 proteins of an alpha-virus, provided that the region which encodes the capsid protein lacks a nuclear targeting signal.
- 25 5. A nucleic acid according to any one of the preceding claims wherein the alpha-virus is a Venezuelan Equine Encephalitis Virus (VEEV).
- 30 6. A deletion mutant of an alpha-virus, which mutant lacks a nuclear targeting signal from the capsid gene.
7. A deletion mutant of an alpha-virus which lacks a region corresponding to nucleotides 7749-7887 of VEEV.
- 35 8. A deletion mutant according to claim 7 wherein the alpha-virus is VEEV.
9. A nucleic acid which encodes a deletion mutant of an alpha-virus as claimed in any one of claims 6 to 8.

10. A recombinant adenovirus which comprises a nucleic acid according to any one of claims 1 to 5 or 9.
- 5 11. A recombinant vaccinia virus which comprises a nucleic acid according to any one or claims 1 to 5 or 9.
12. A plasmid which comprises a nucleic acid according to any one or claims 1 to 5 or 9 under the control of a
10 promoter which allows expression thereof.
13. A vaccine which comprises a virus according to claim 10 or claim 11 or a plasmid according to claim 12.
- 15 14. A vaccine according to claim 13 which further comprises pharmaceutically acceptable carrier.
15. A method of producing a protective immune response to an alpha-virus, said method comprising administering to a
20 mammal a vaccine according to claim 13 or claim 14.

Fig.1.

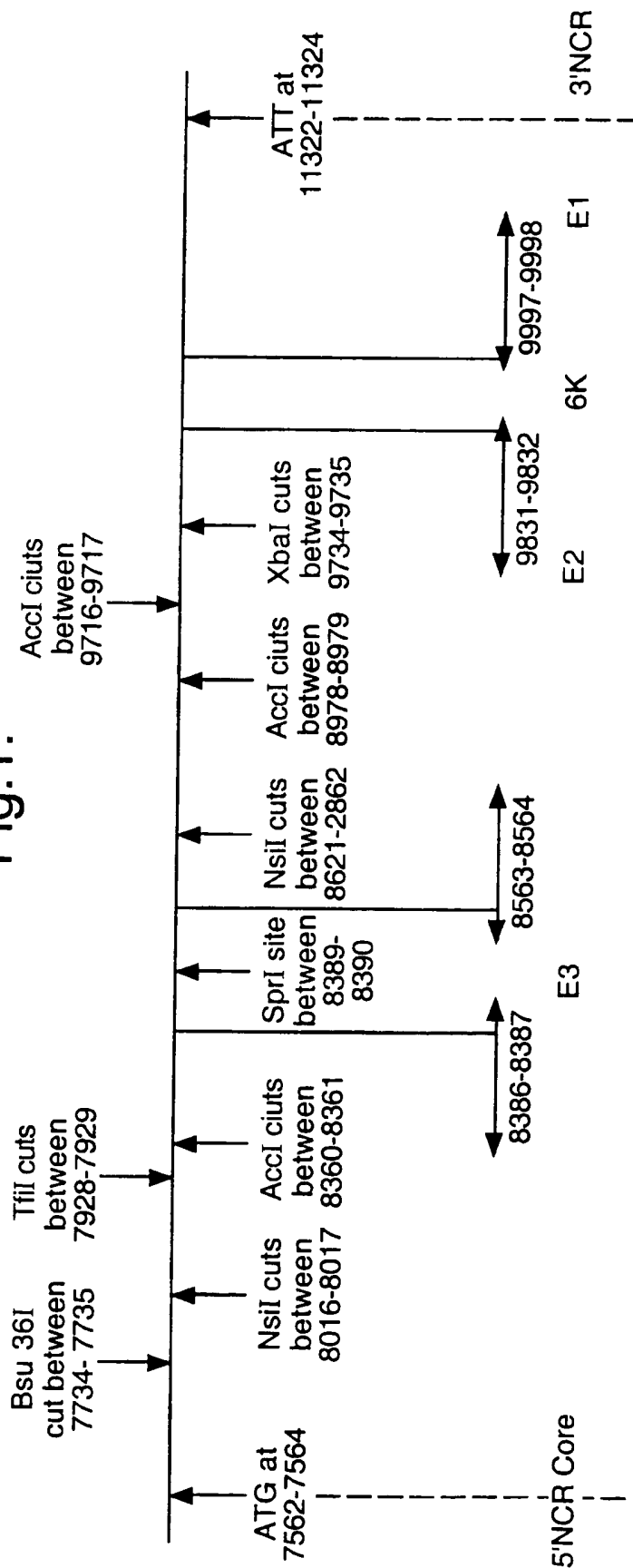


Fig.2a.

L1 CTAGAG**GAATTCT**
 _____**TCTTAAGAGATC**
 XbaI EcoRI XbaI

Fig.2b.

L4A CTAG**TGAC**ATGGT**GAATTC**ACCATG**TCA**
ACTGTACCACTTAAGTGGTACAGTGATC
 SpeI START Kozak EcoRI Kozak START SpeI
 ^ ^
 3 nt of 5' of E1 3 nt of 5' of E1

Fig.2c.

L7L8 GATCCG**TATAC**TCTAGAG****GCATG
 GCATATGAGATCTC
 BamHI *AccI* XbaI SphI

Fig.2d.

L5L6 ATACTCCGGAGAACTGCGAGCAATGGTAAT
 TGAGGCCTCTTGACGCTCGTTACCATTAGATC
 AccI 3' Core *STOP* **XbaI**

Fig.2e.

L10L13 TTAATCTAGAAGATGCA
 ACGTAATTAGATCTTCT
 NsiI STOP XbaI Start NsiI

Fig.2f.

L28L29 TGAGGGGCCATCCGCT AACAAAGAAACCAGGCAAGAGACAGC..
 _____CCCCGGTAGGCGA TTGTTCTTTGGTCCGTTCTCTGTCTG

Bsu 36I

nt7735-7750--Λ--

nt7886-8024

..GCATGGTCATGAAATTGG
 CGTACCAGTACTTTAACCTTA

TfiI

Fig.2g.

L30L31 ATACTCCGGAGAACTGCGAGCAATGGTCACTAGTA
 _____TGAGGCCTCTTGACGCTCGTTACCAGTGATCATTGCA

--Λ--

AccI

Core

E3 *SpeI* *HindIII*

Fig.2h.

P2 GGCCGGATCCGGATGTTCCCGTTCCAGCC

GC Rich tail *BamHI*

Start 5' Core

Fig.2i.

P3 GCGCGGATCCCTCAGGTGGCGCG

GC Rich tail *BamHI* *Bsu 36I* Core

Fig.2j.

L20 GCGCCA CTGAGGGGCCATCCGC

GC Rich tail *Bsu 36I*

5' Nuclear targeting signal

Fig.2k.

L21 CGGGGATTCTGGTTTCTTGTTGGTCTTCTTCTTG

GC Rich tail *TfiI*

3' Nuclear targeting signal

Fig.3.

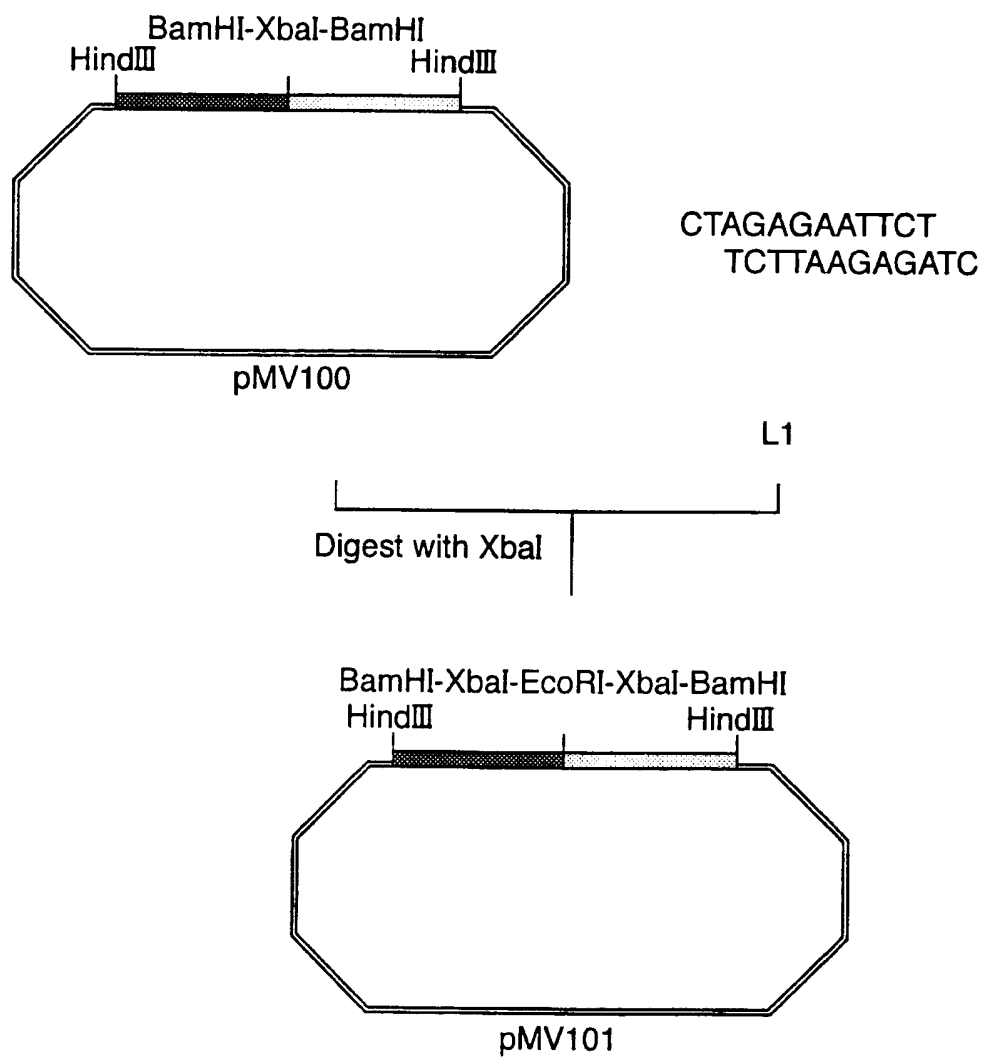


Fig.4.

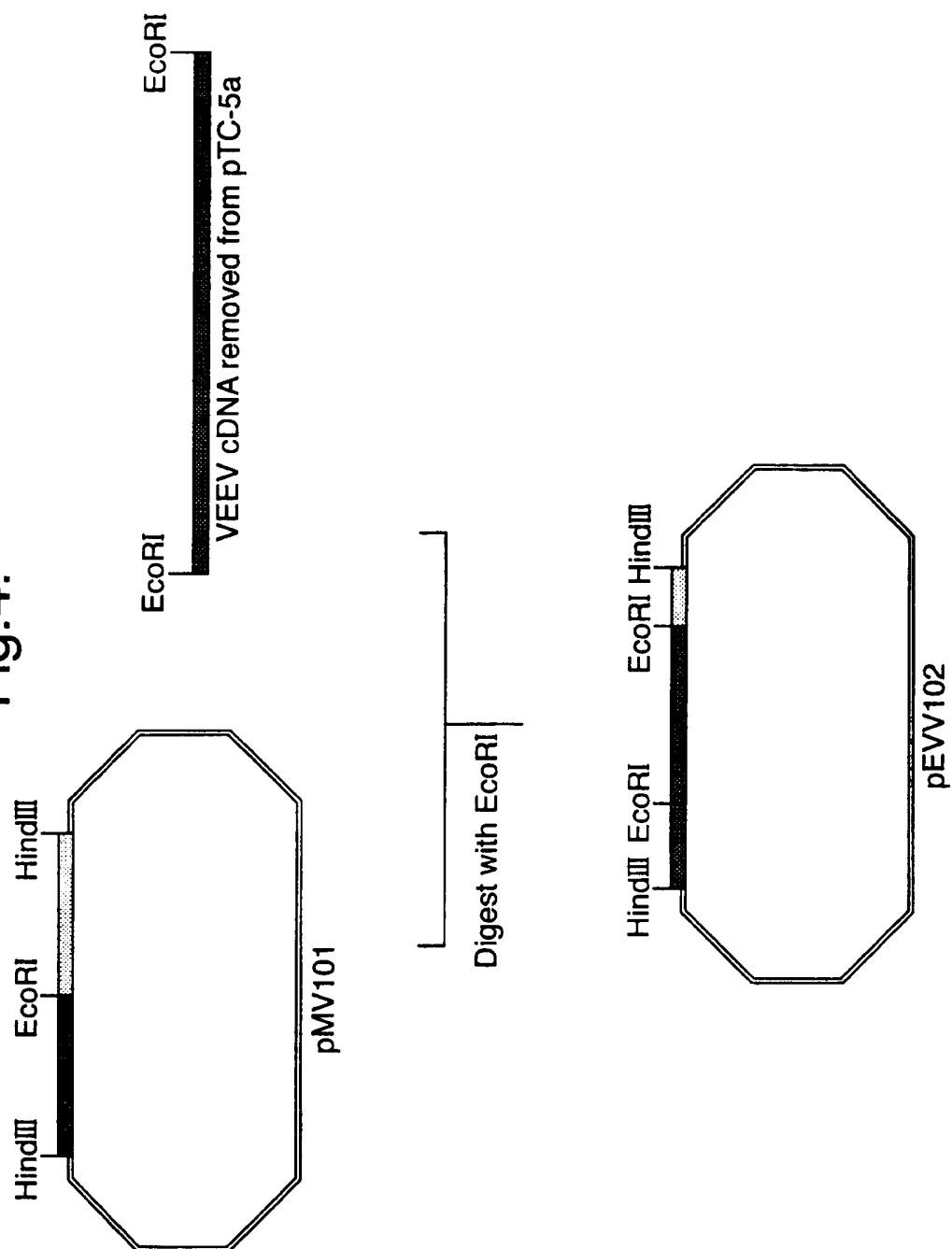
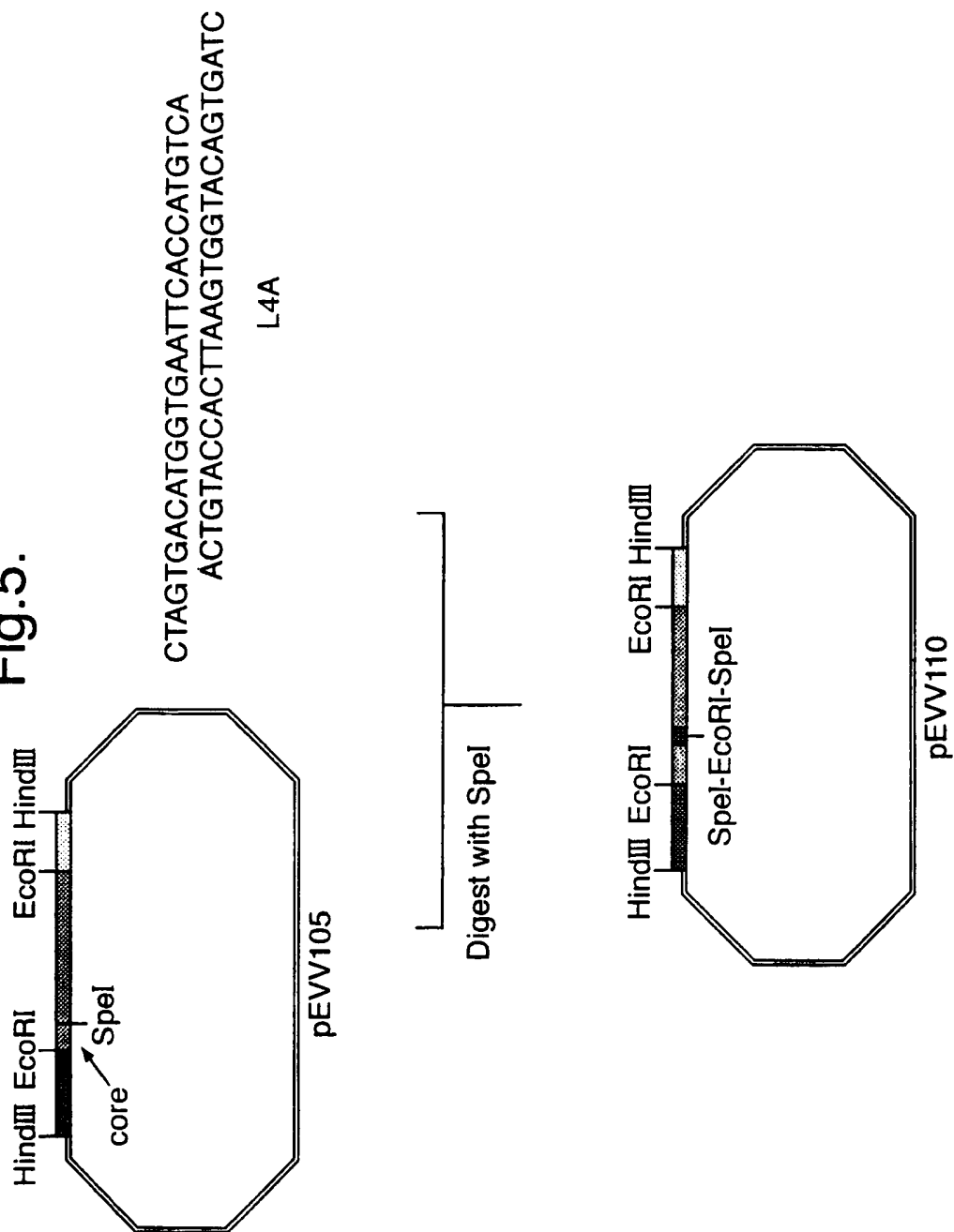


Fig.5.



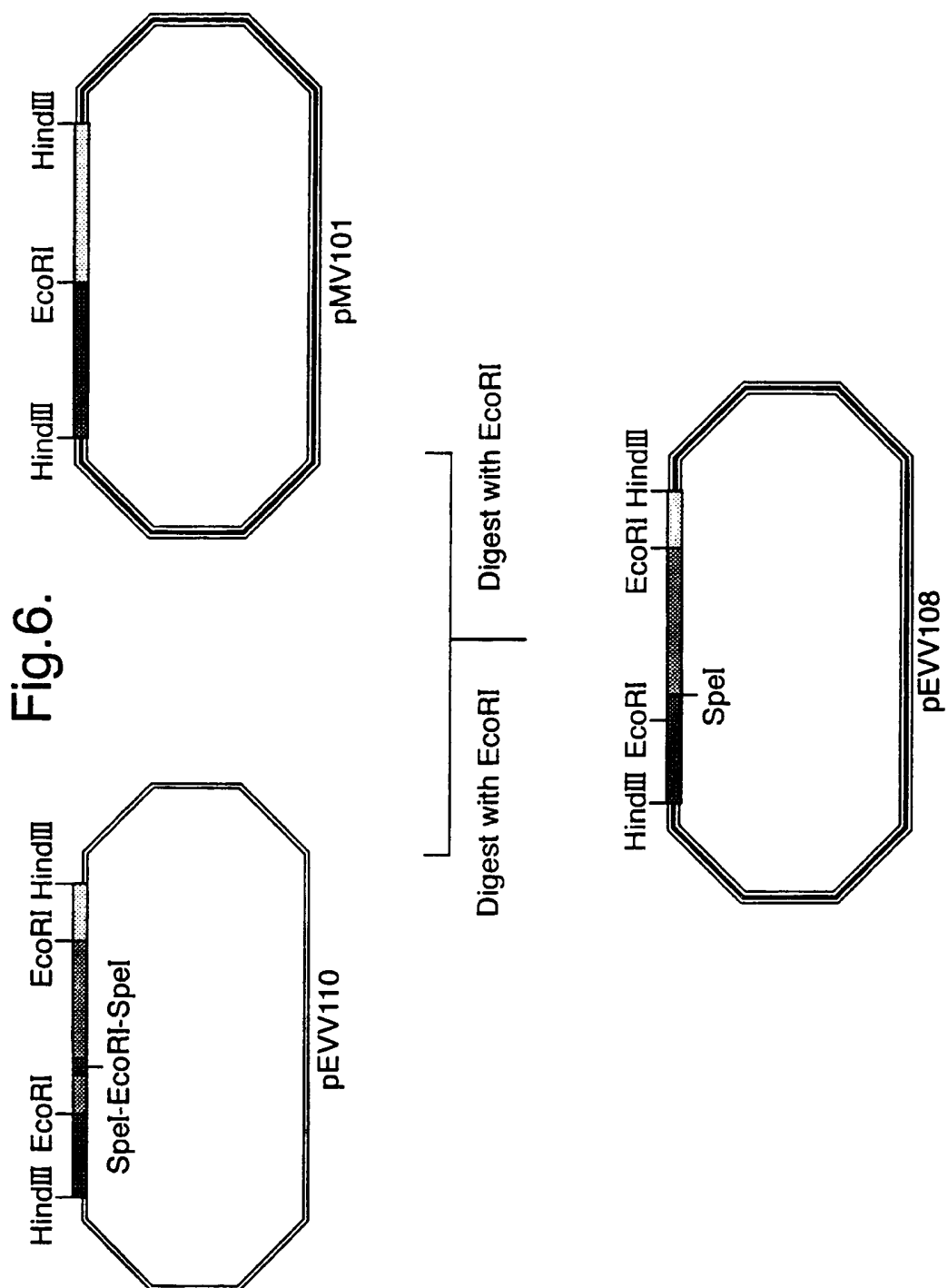
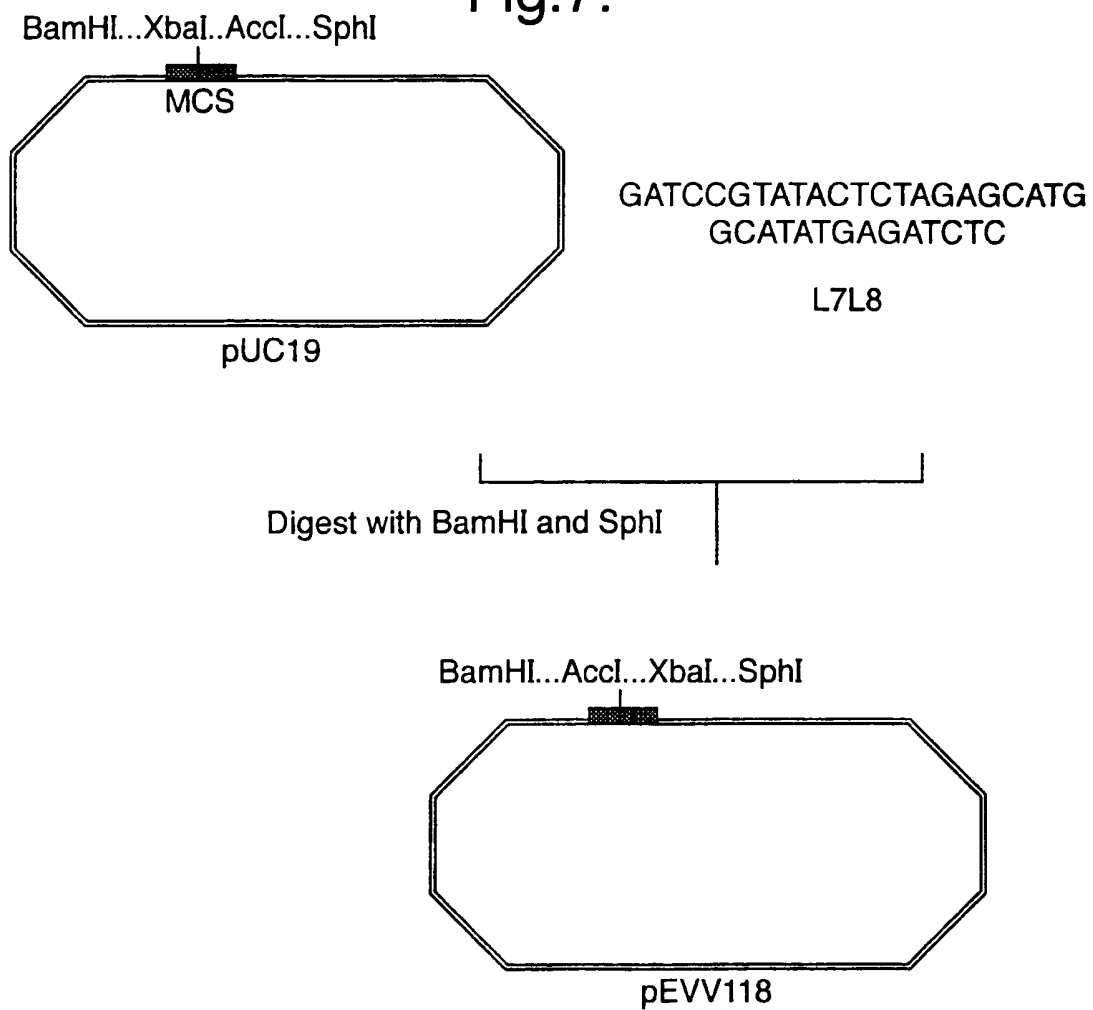
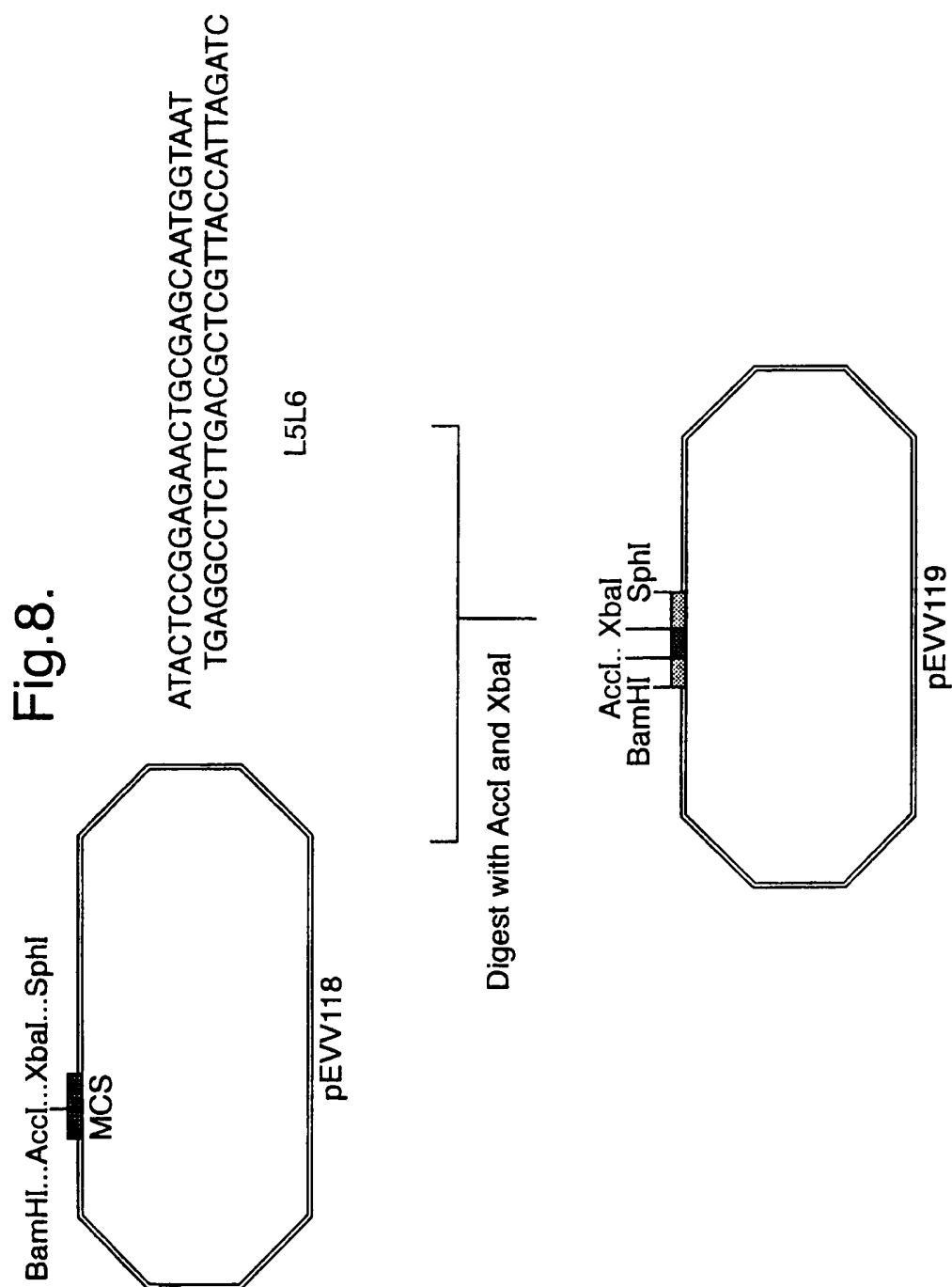
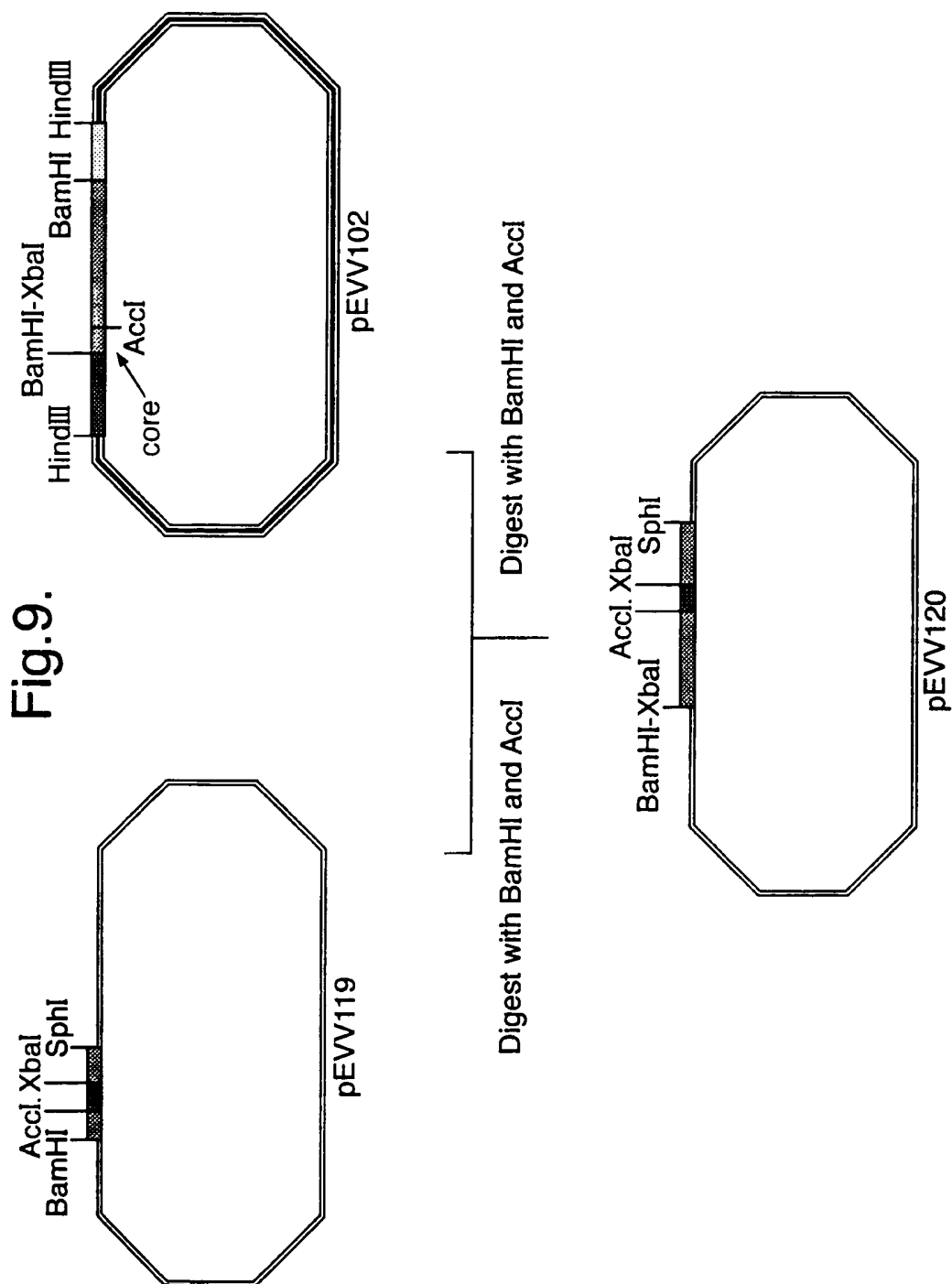


Fig.7.







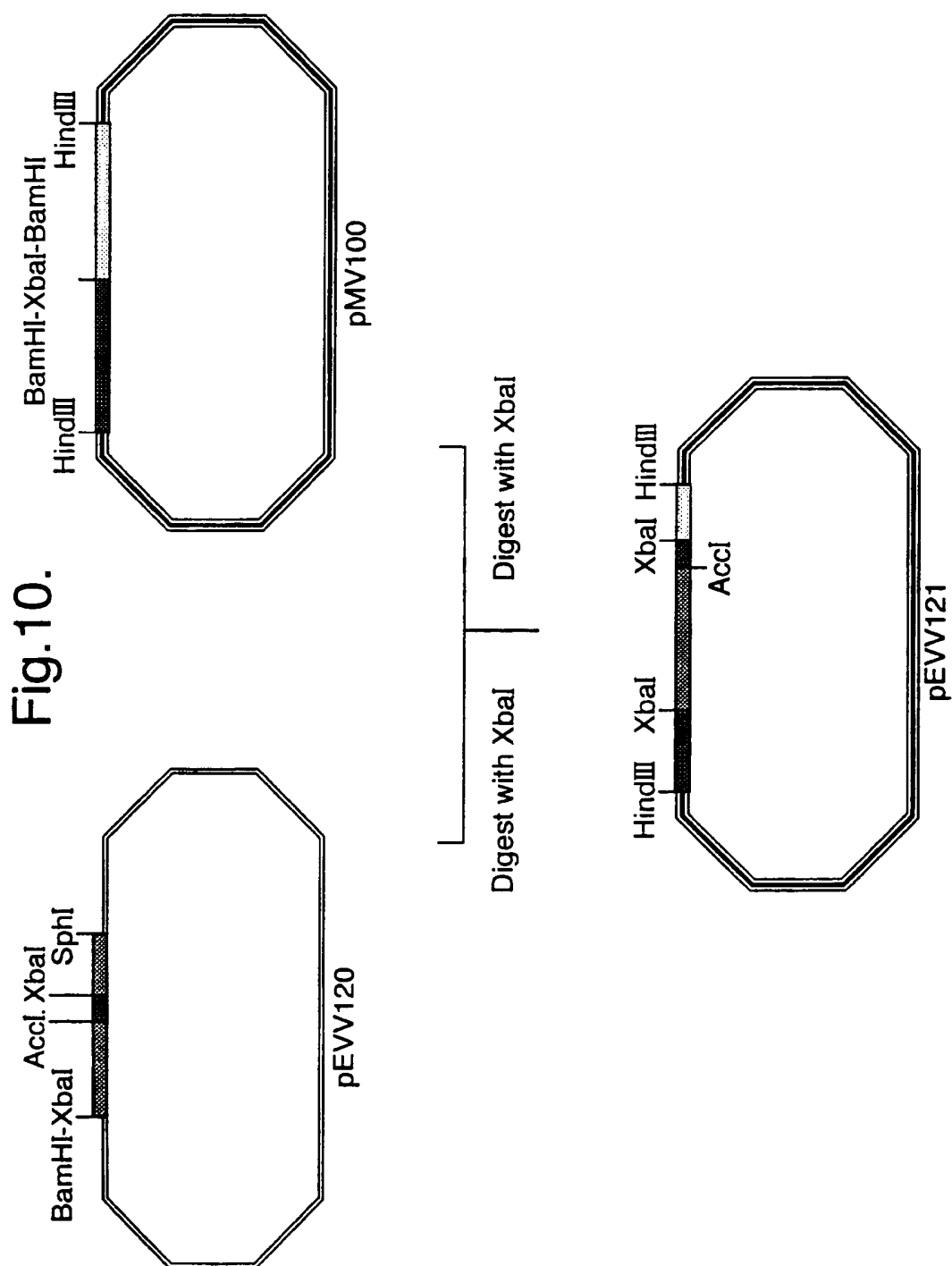
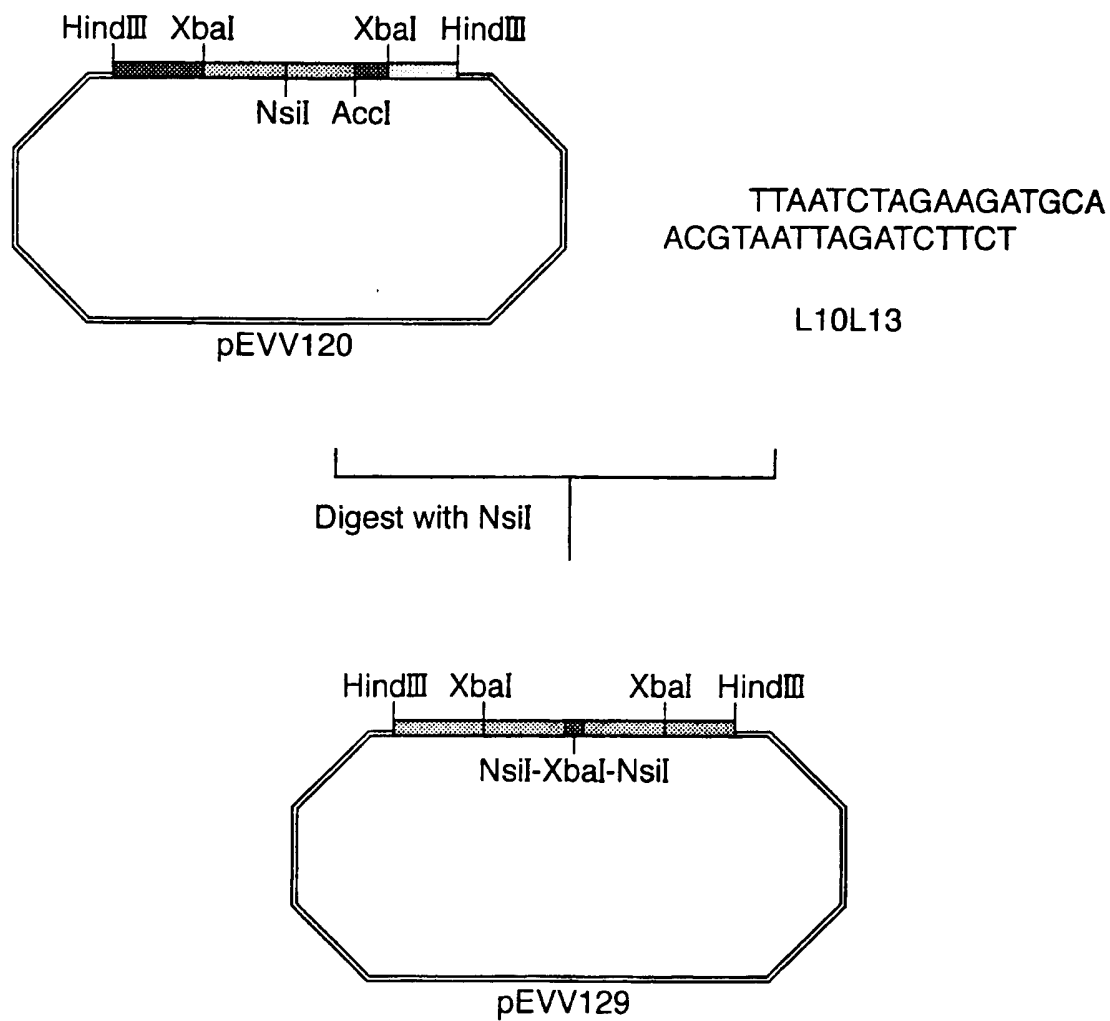


Fig.11.



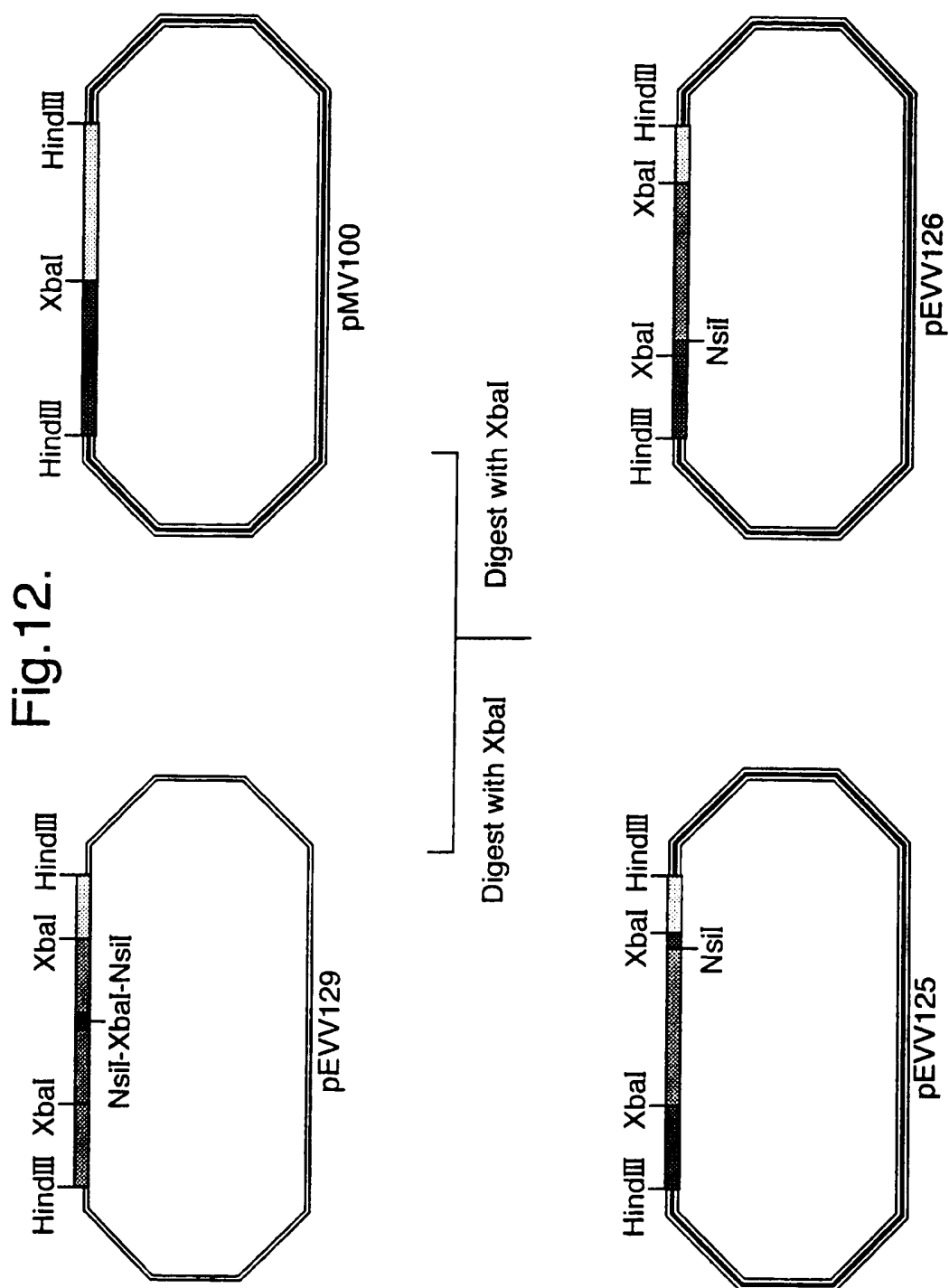
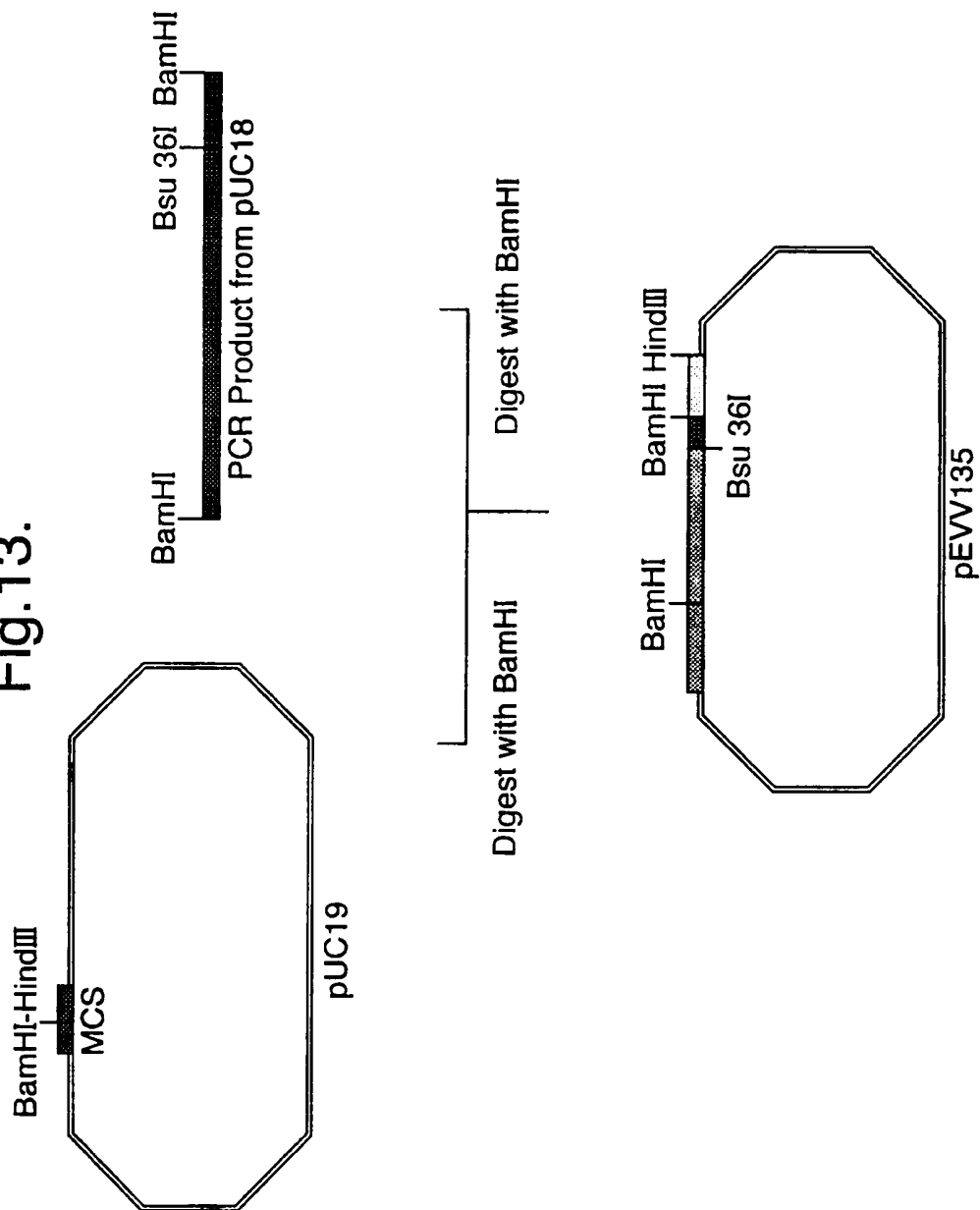


Fig.13.



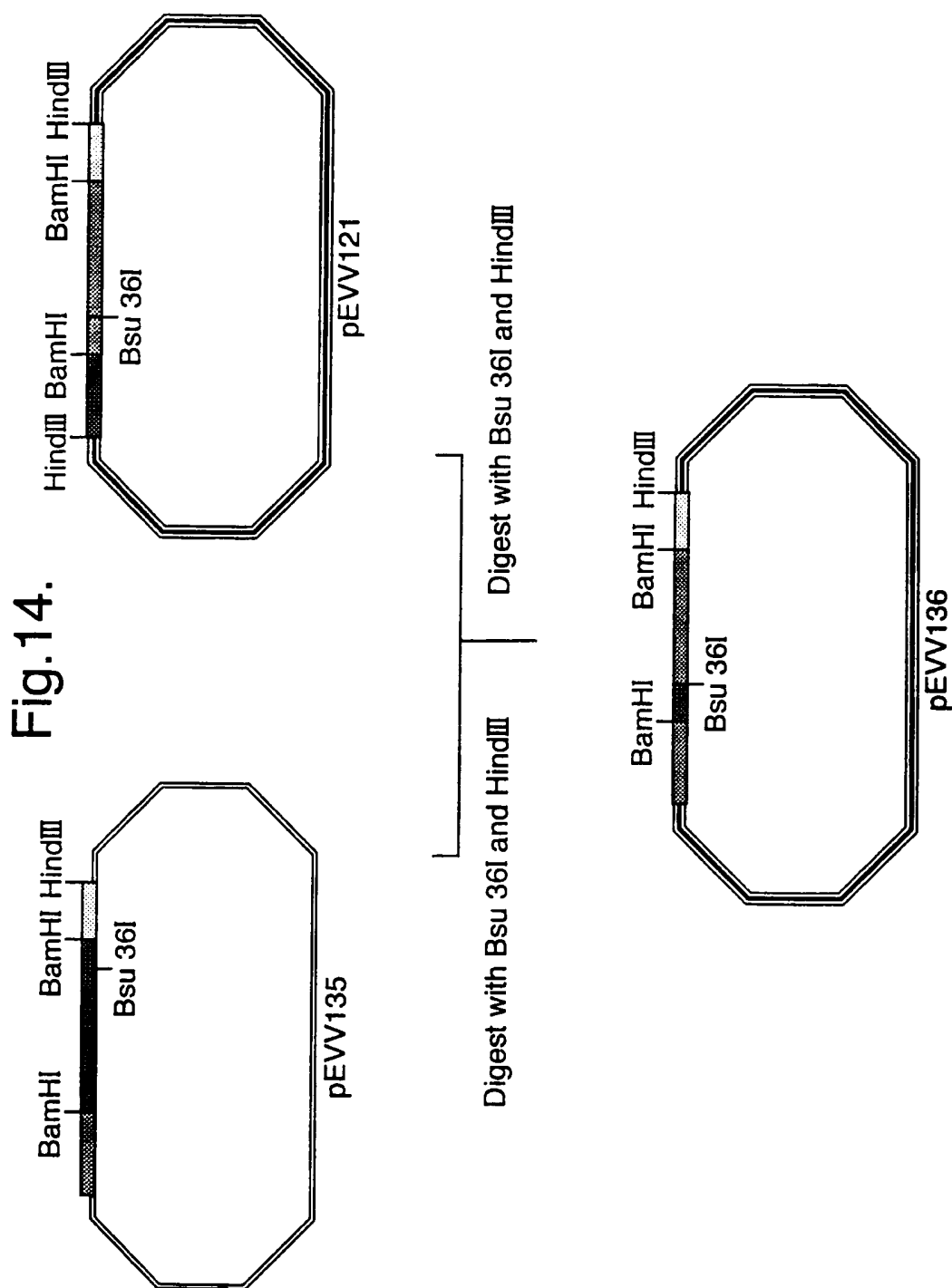


Fig.15.

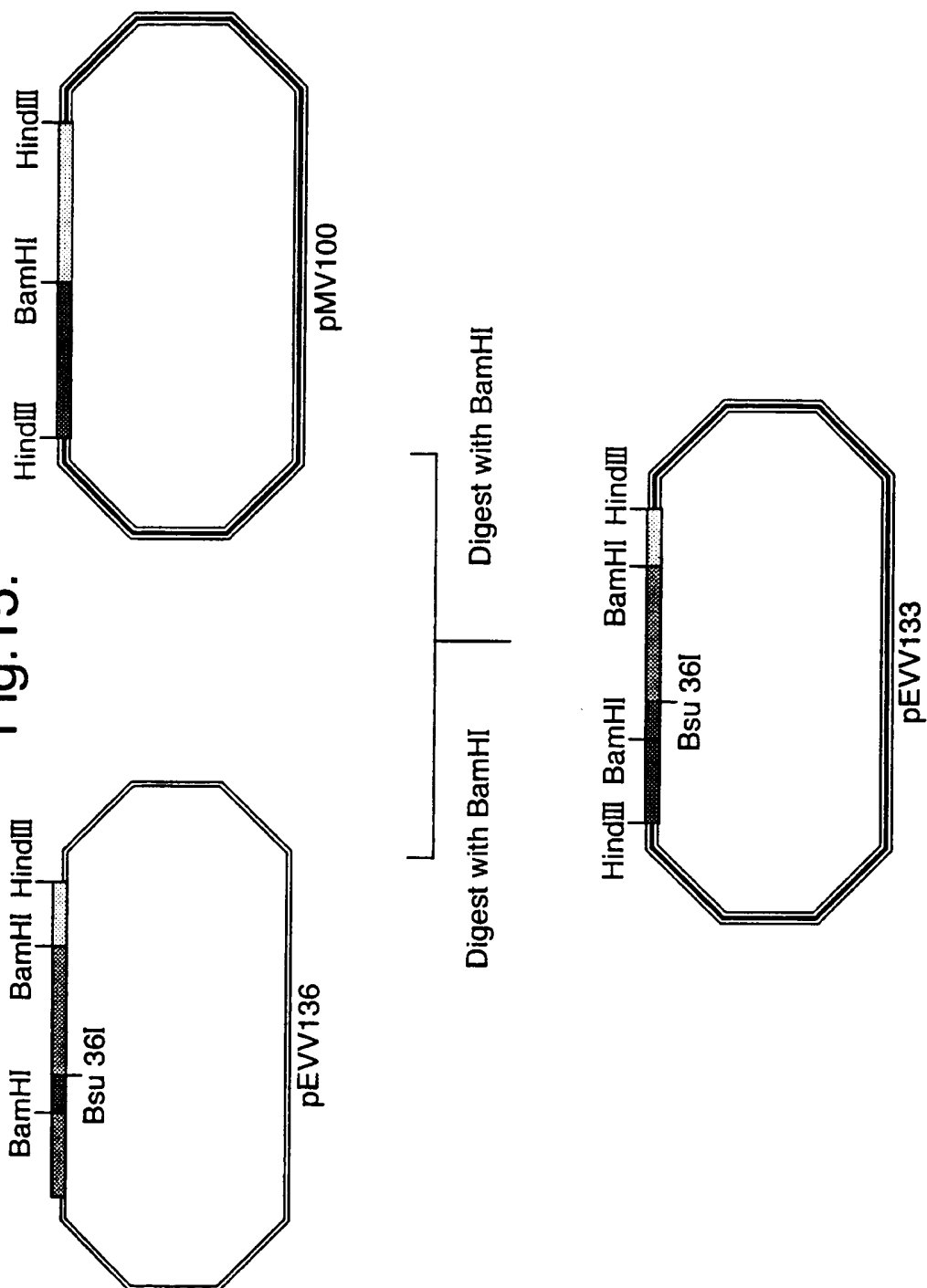


Fig.16.

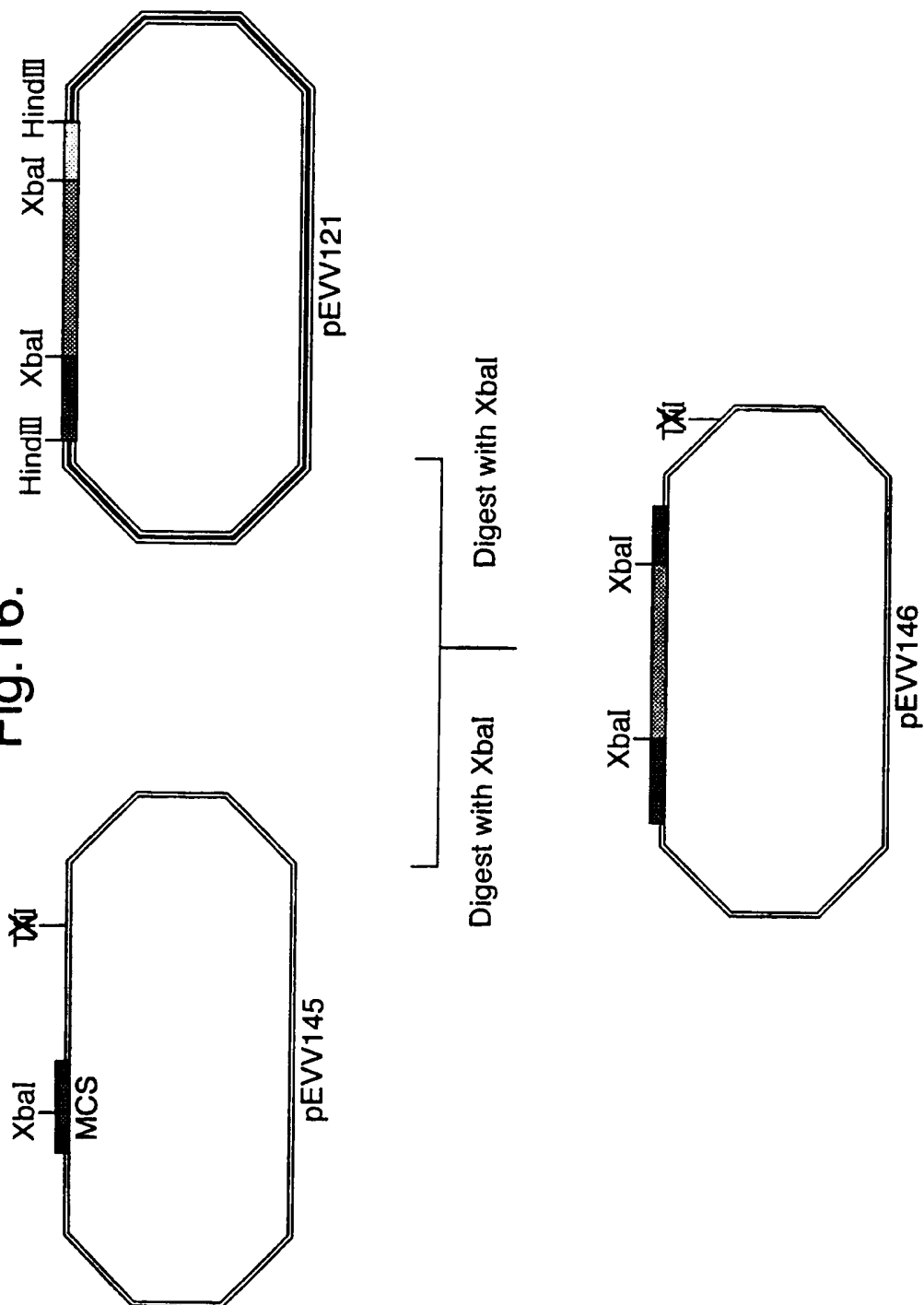


Fig.17.

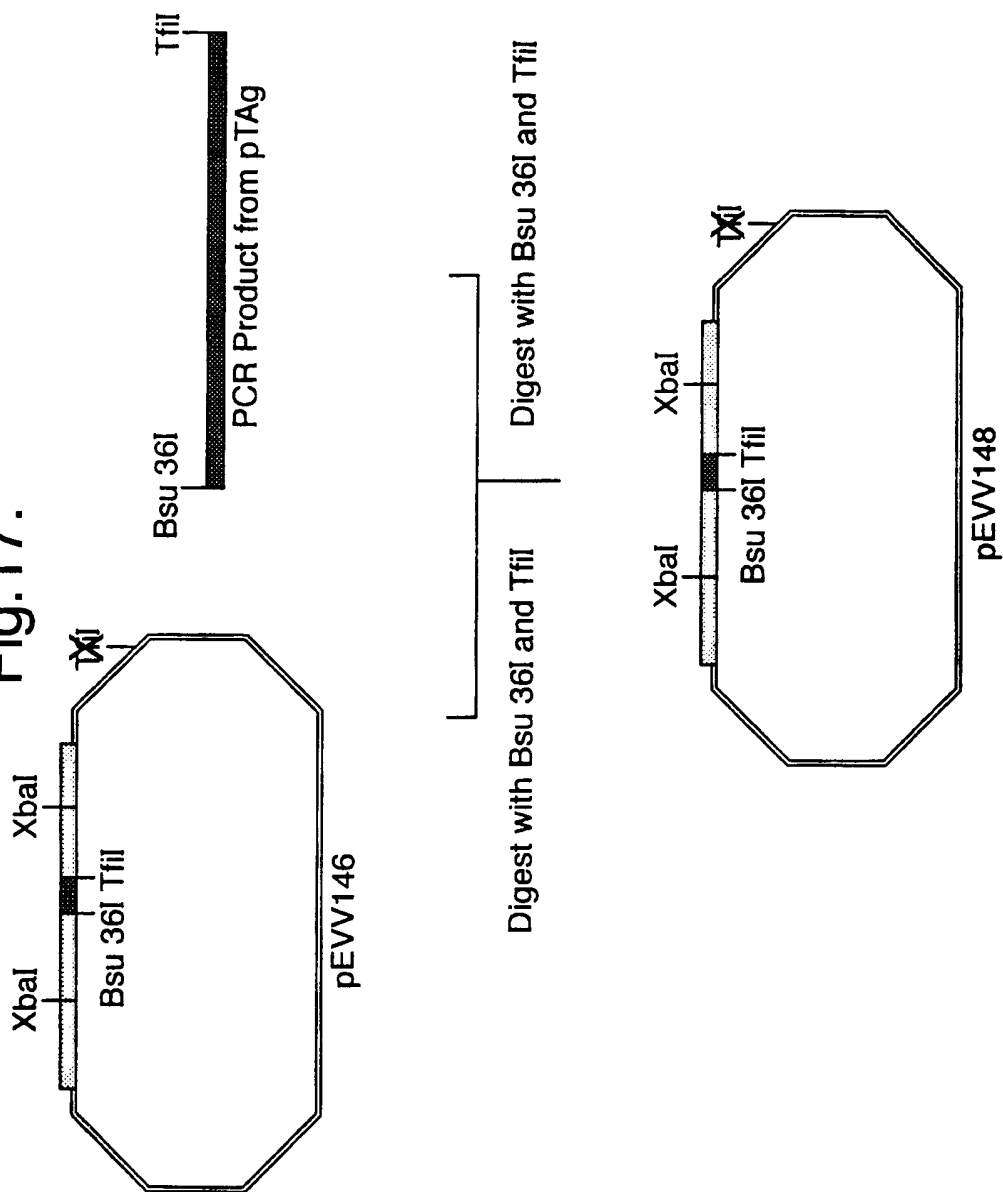
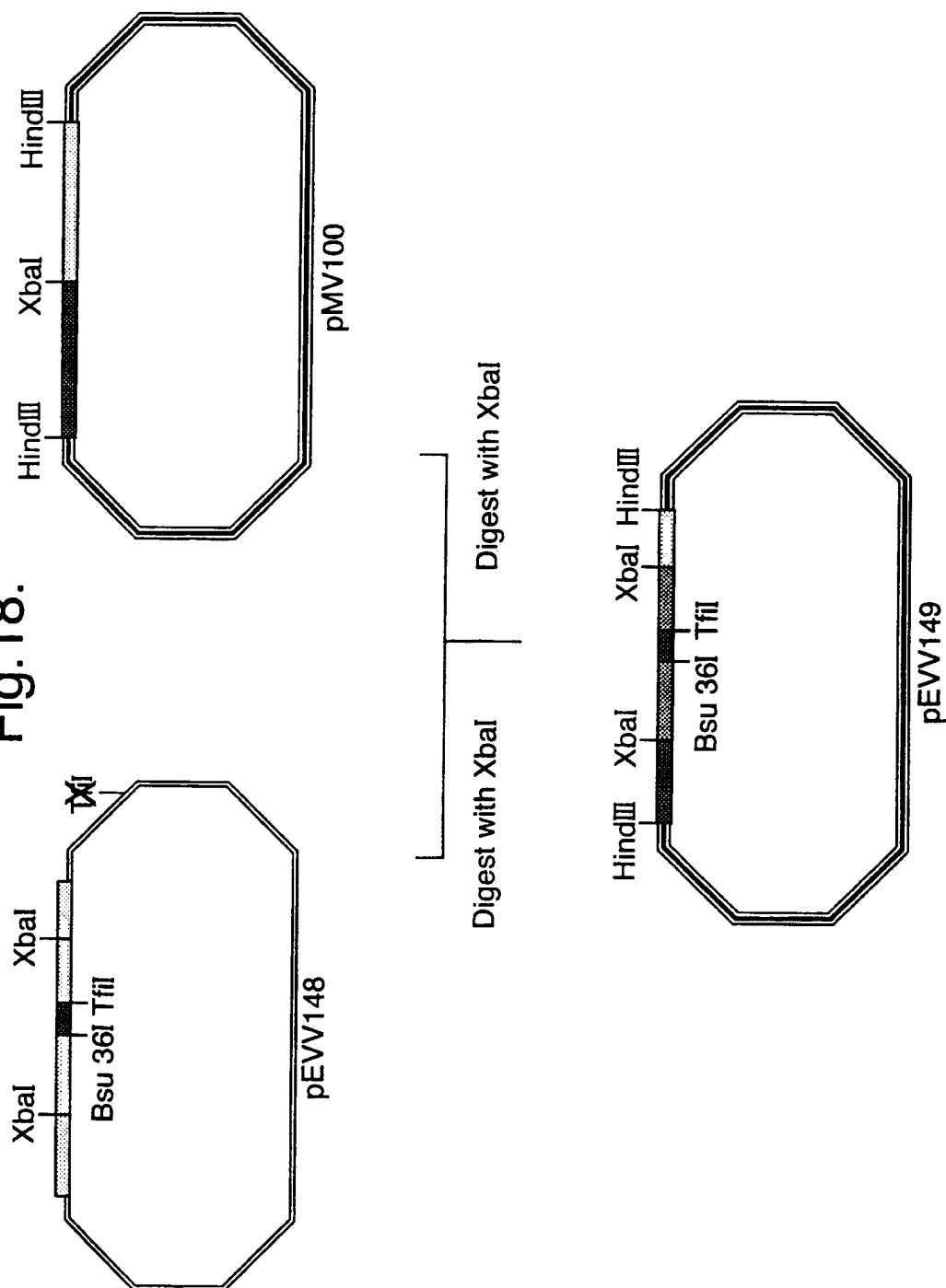


Fig. 18.



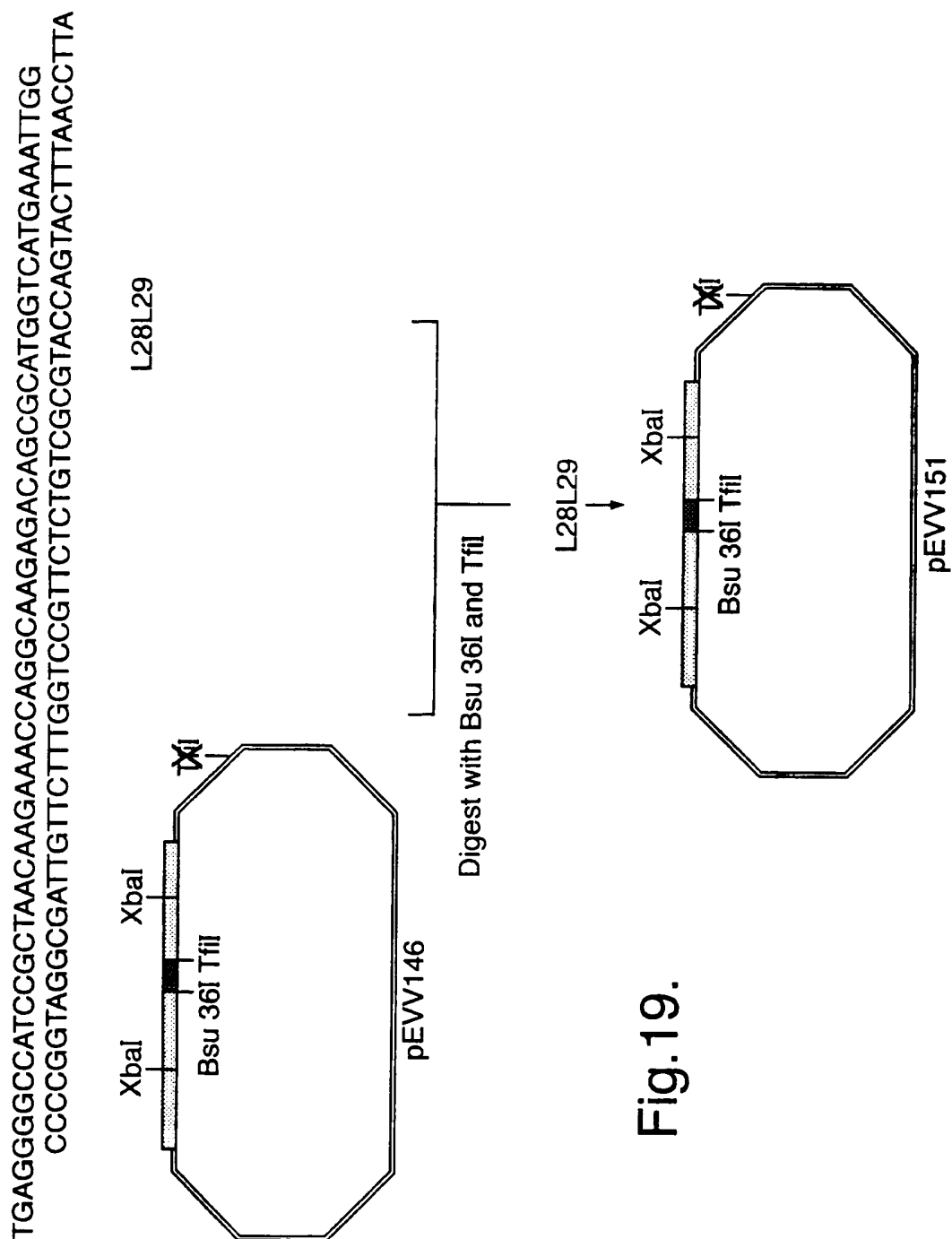
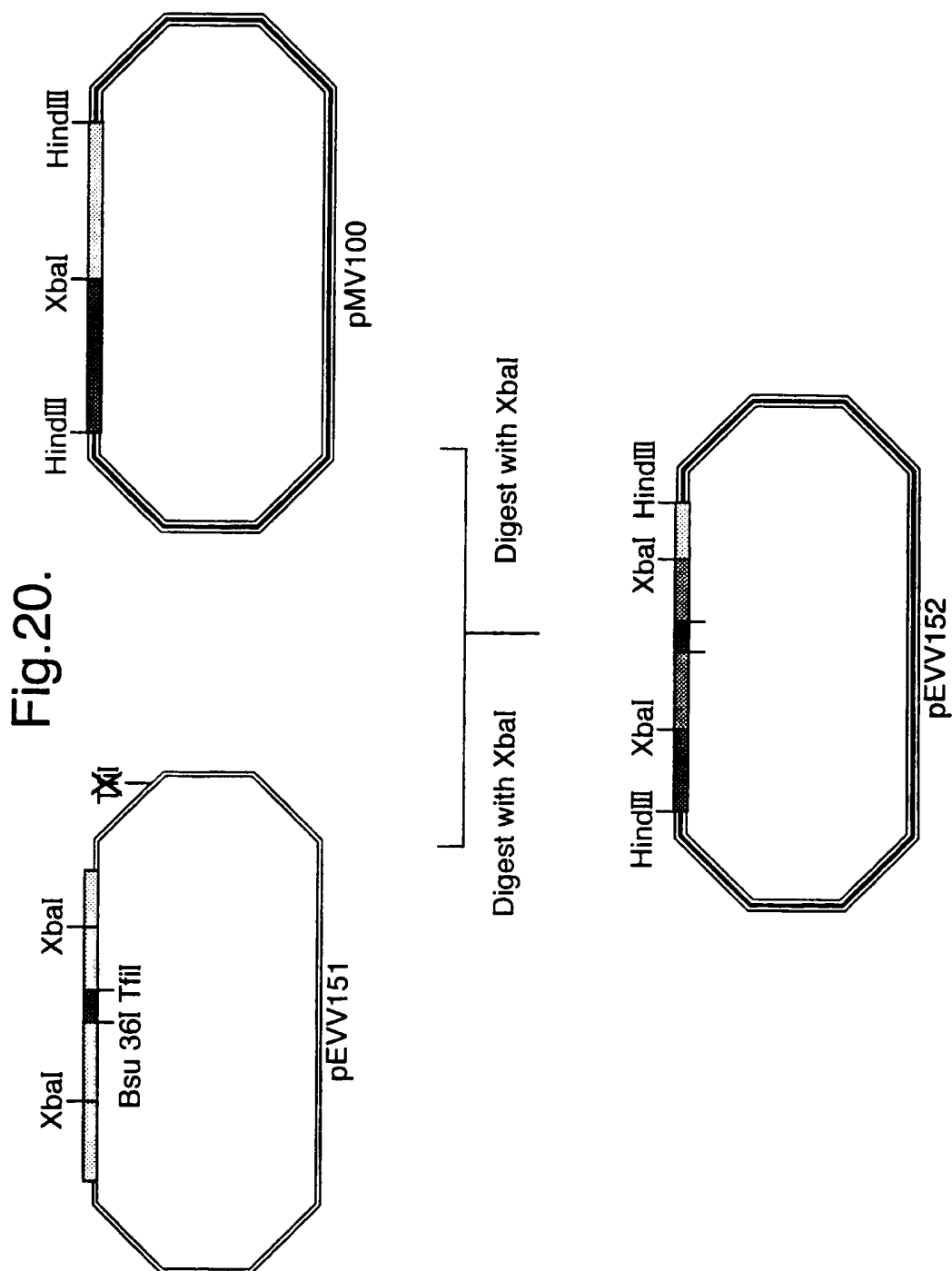


Fig.19.



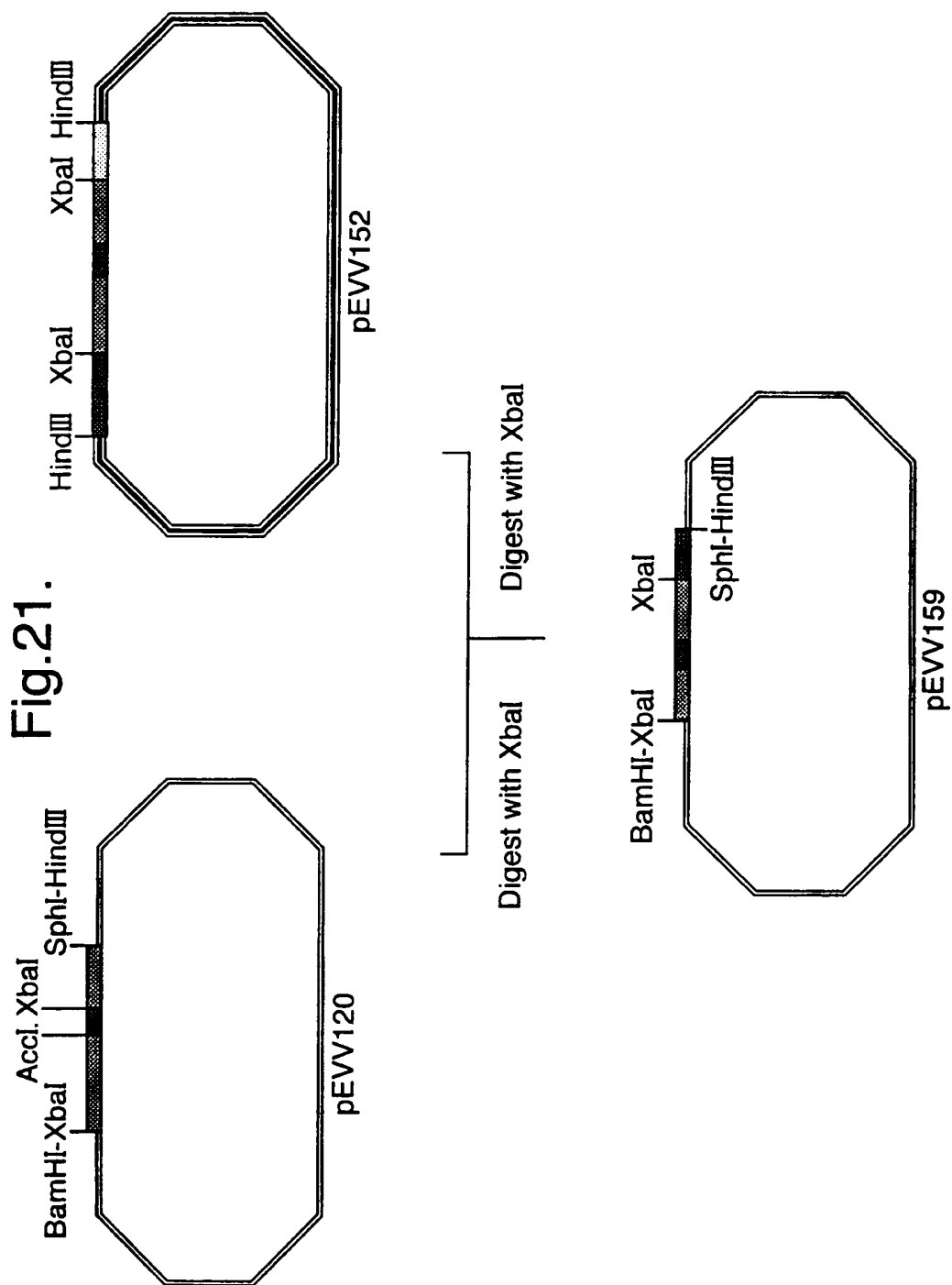


Fig.22.

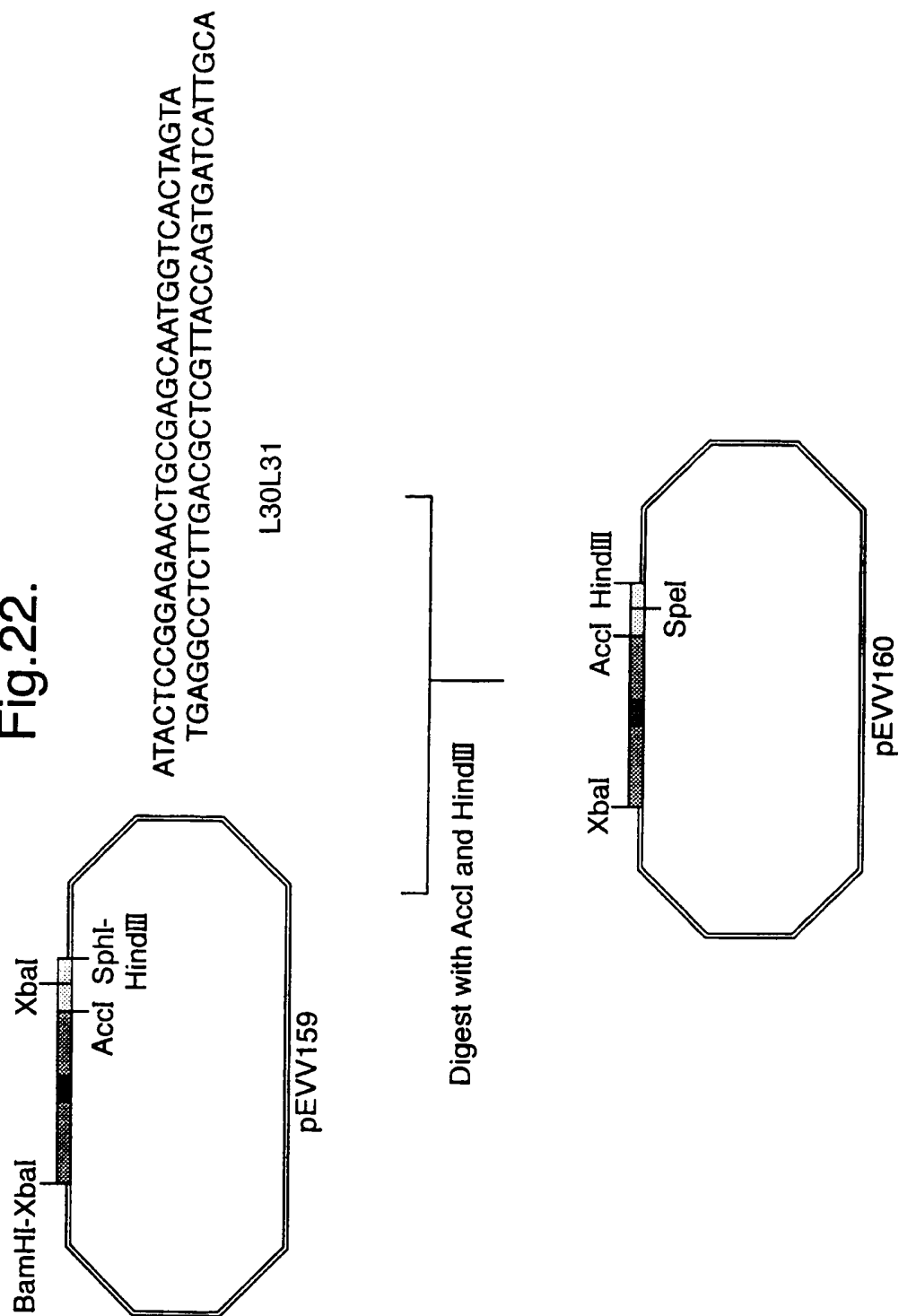
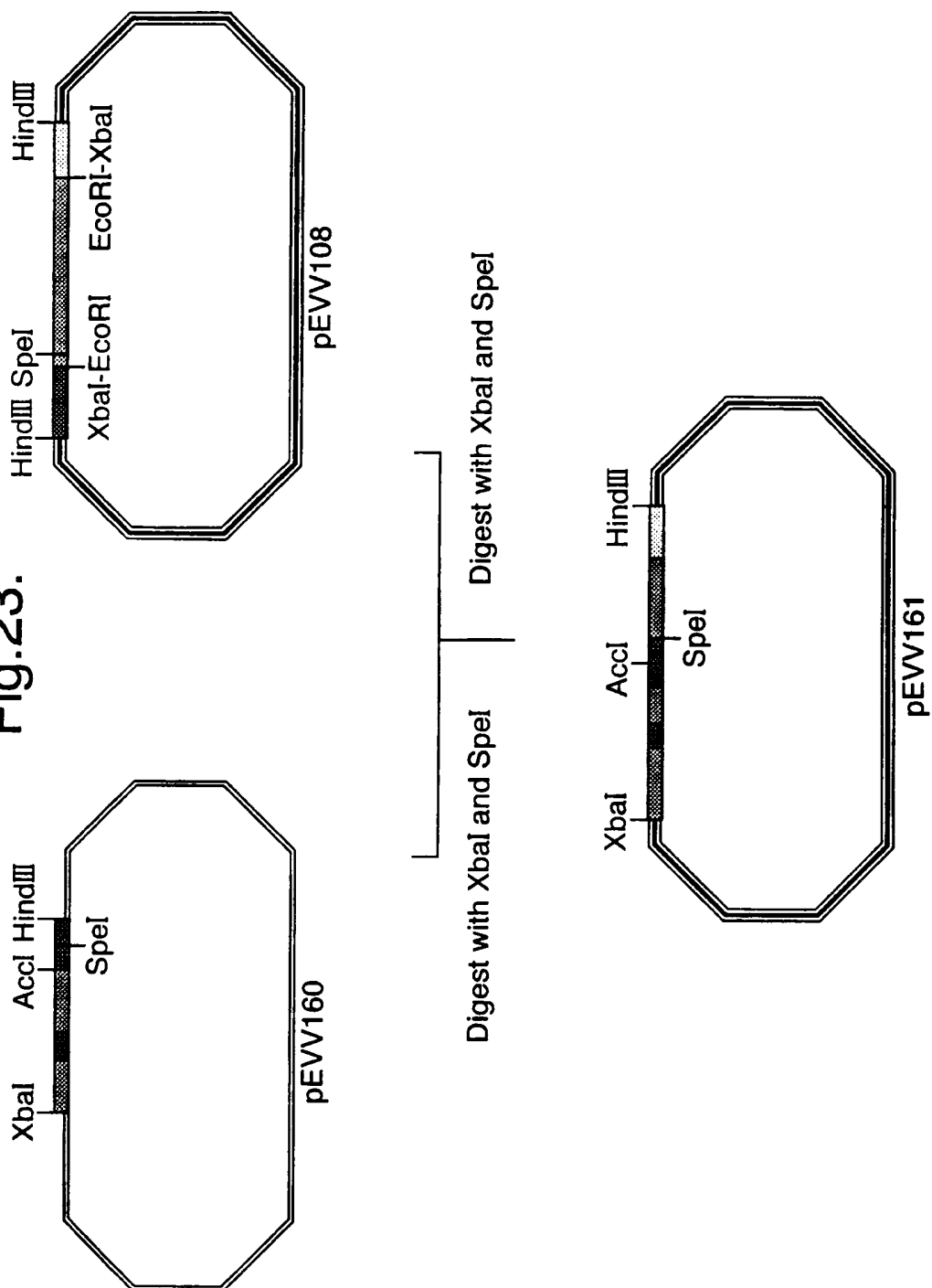


Fig.23.



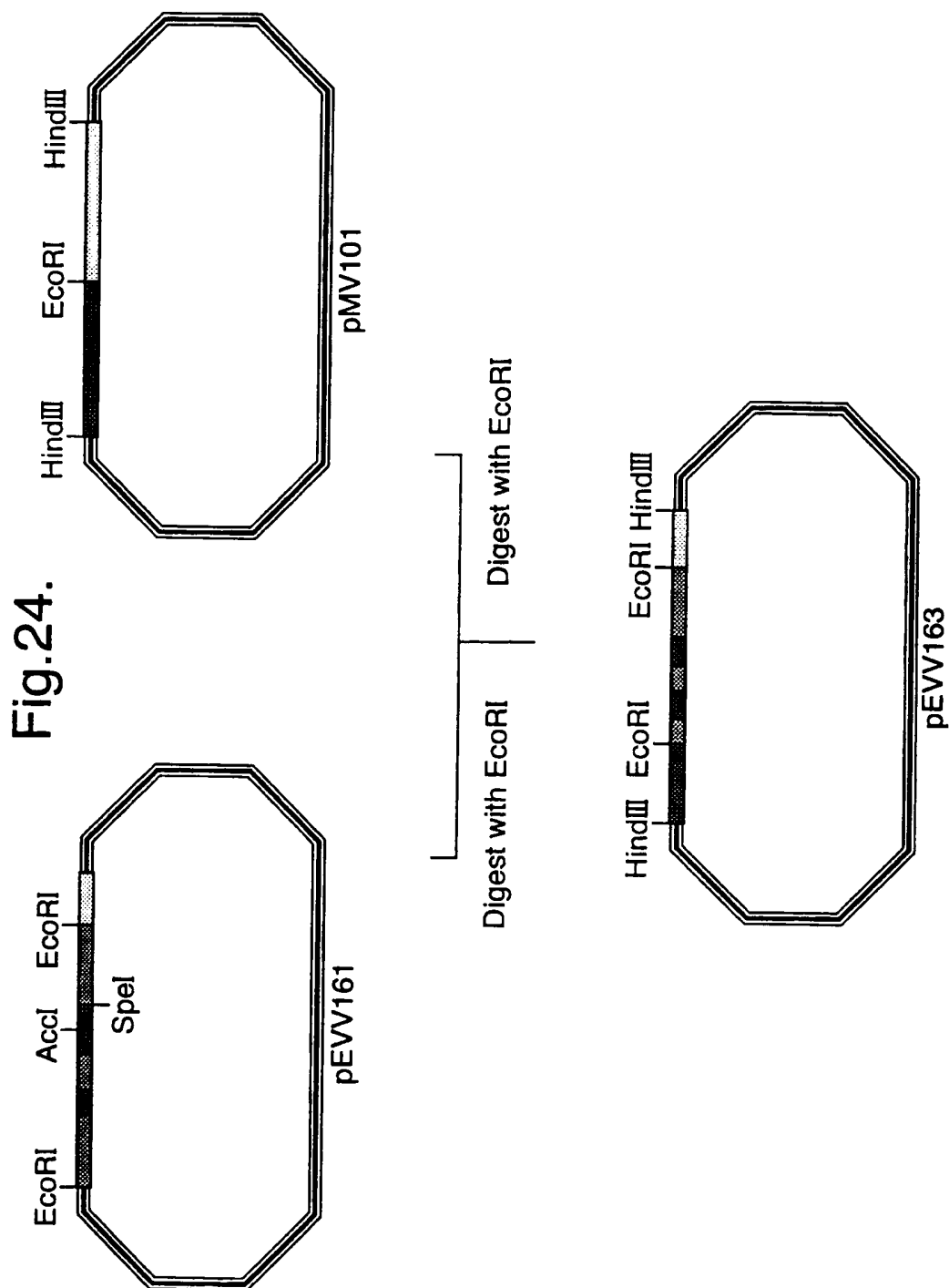


Fig.25.

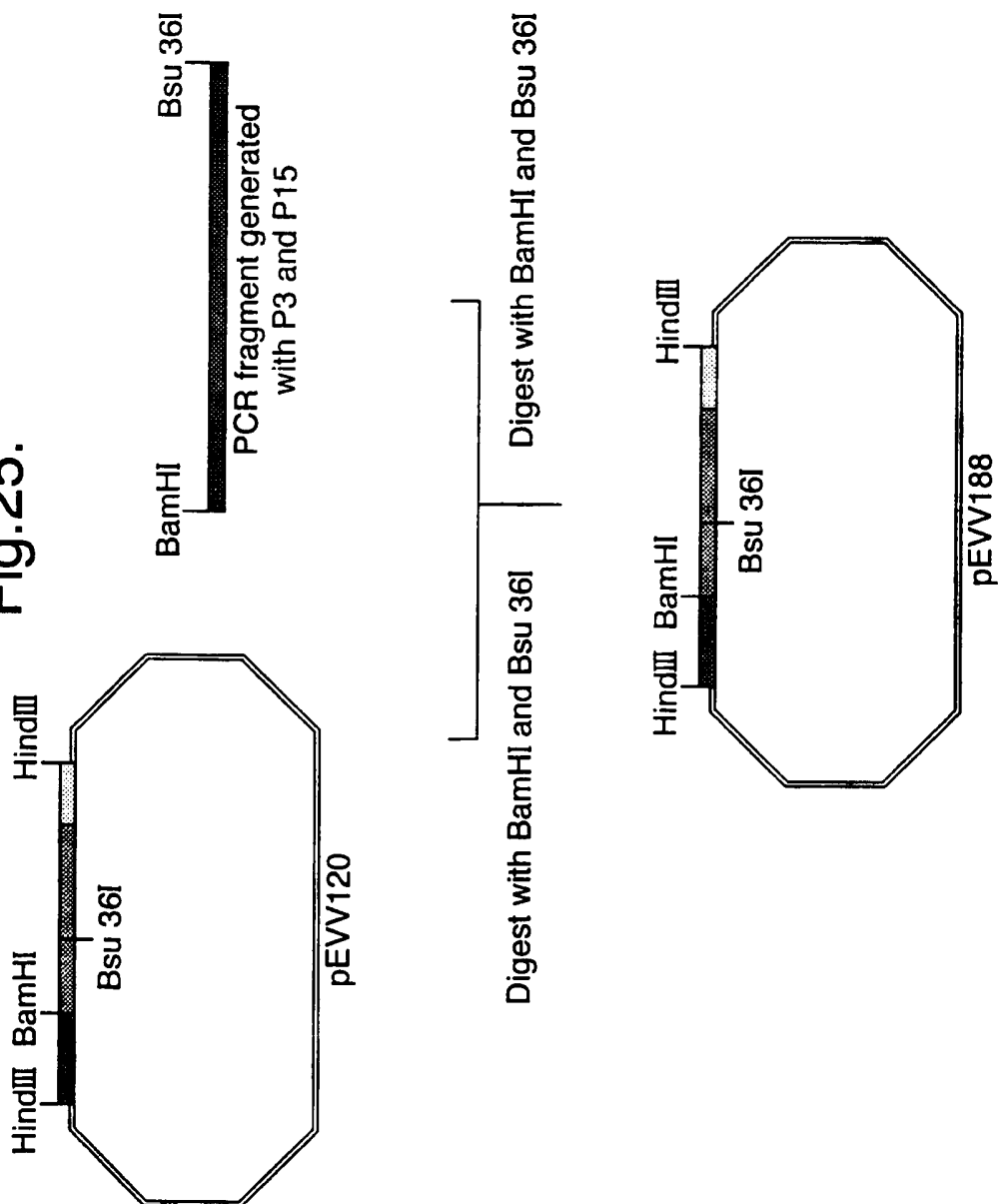


Fig.26.

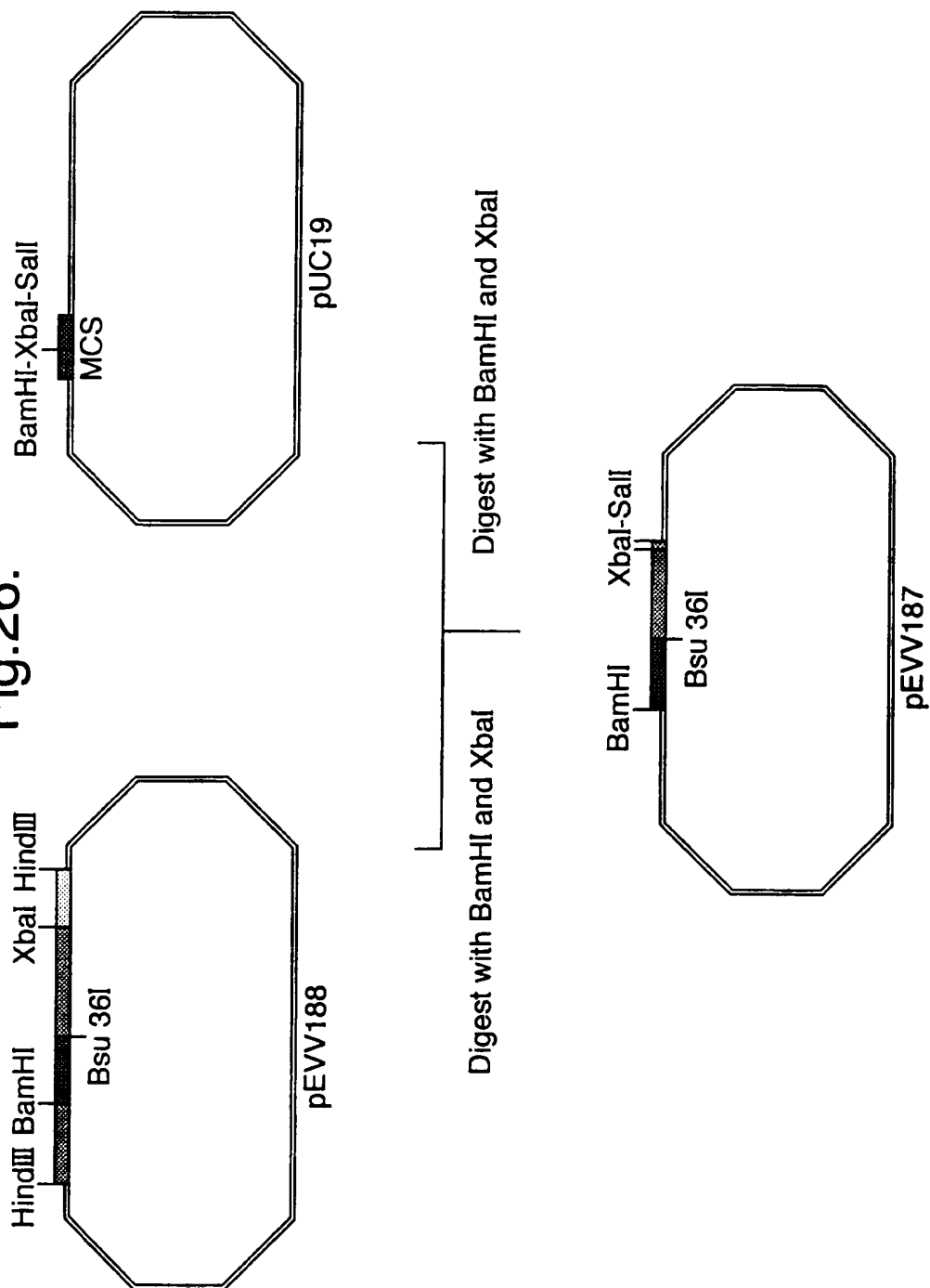


Fig.27.

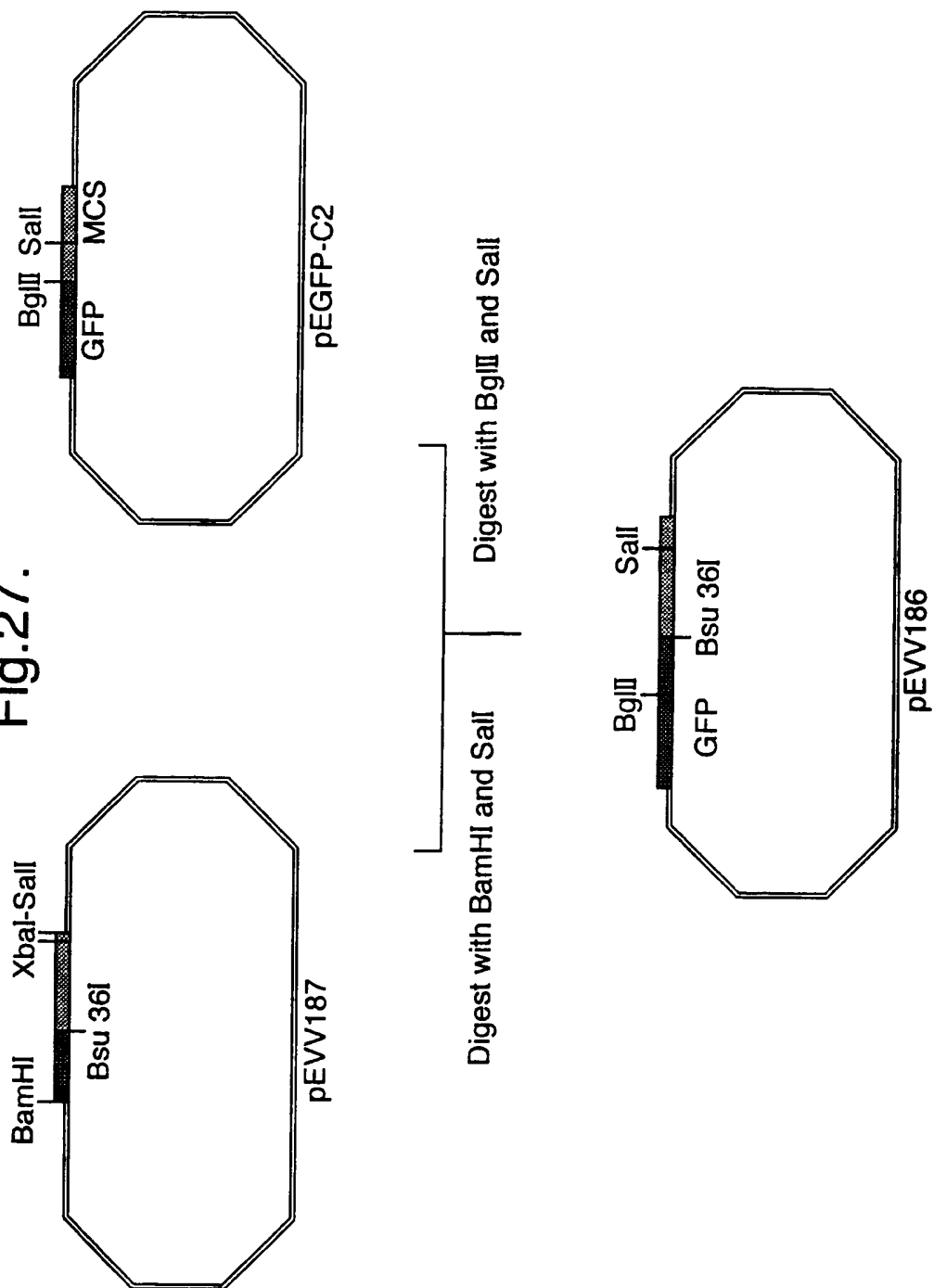
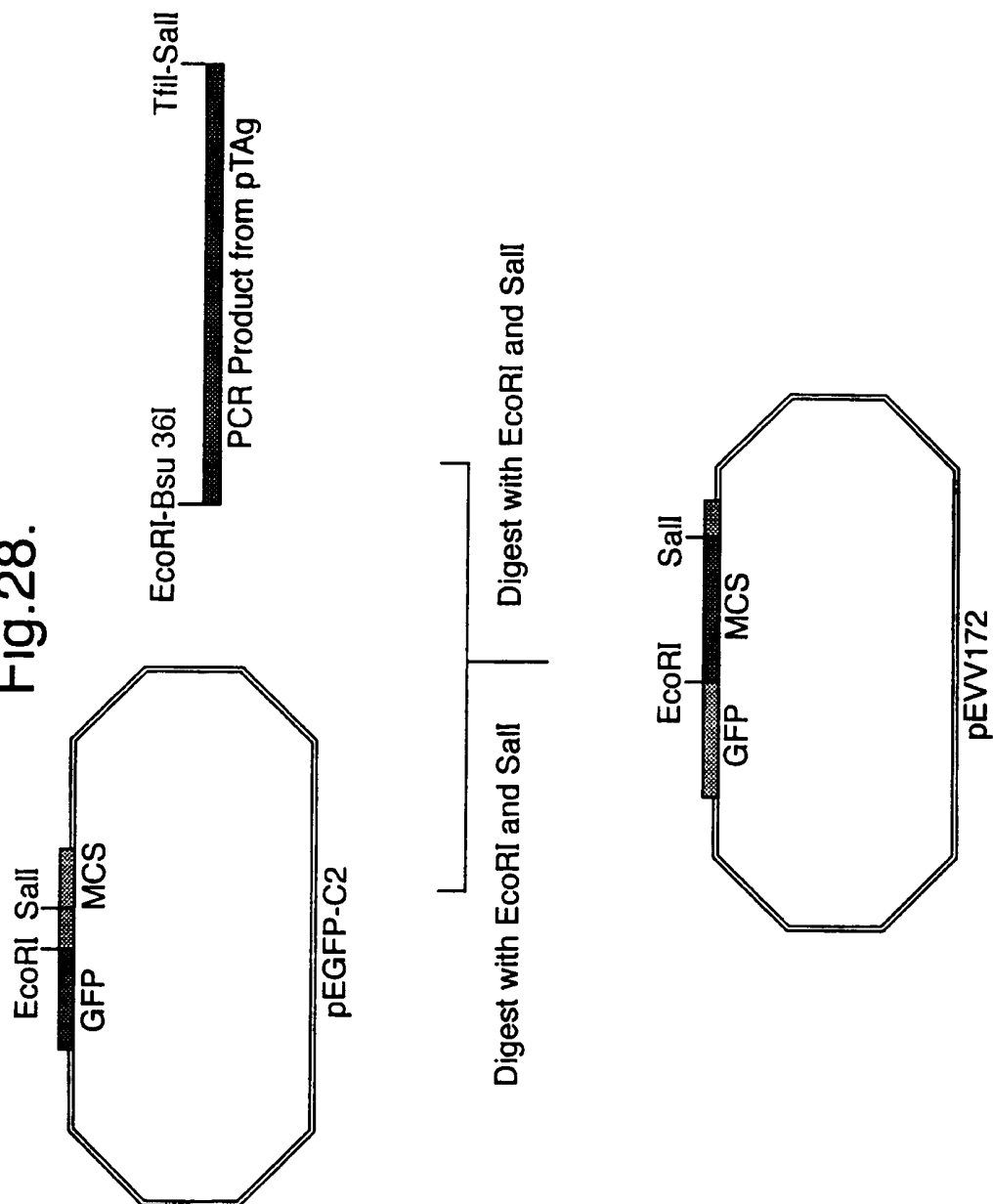


Fig.28.



INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/00874

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/18 C12N15/33 A61K39/193

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	WO 98 53077 A (WALTER REED ARMY INST RES) 26 November 1998 (1998-11-26) abstract page 11, line 1 - page 12, line 19 page 19, line 33 - page 20, line 14 ---	1-15
Y	US 5 185 440 A (JOHNSTON ROBERT E ET AL) 9 February 1993 (1993-02-09)	1,3-5
A	the whole document ---	10-15
Y	JAKOB R.: "Nucleolar accumulation of core protein in cells naturally infected with Semliki Forest virus" VIRUS RESEARCH, vol. 30, no. 2, 1993, pages 145-160, XP002110934 cited in the application the whole document ---	1,3-5
-/--		

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

26 August 1999

Date of mailing of the international search report

09/09/1999

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INTERNATIONAL SEARCH REPORT

Inter. nal Application No

PCT/GB 99/00874

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>KINNEY R.M. ET AL.: "The full-length nucleotide sequences of the virulent trinidad donkey strain of venezuelan equine encephalitis virus and its attenuated vaccine derivative strain TC-83"</p> <p>VIROLOGY, vol. 170, no. 1, 1989, pages 19-30, XP002110935 ORLANDO US cited in the application the whole document</p> <p>----</p>	1-15
A	<p>KUHN R. ET AL.: "Attenuation of Sindbis virus neurovirulence by using defined mutations in nontranslated regions of the genome RNA"</p> <p>JOURNAL OF VIROLOGY, vol. 66, no. 12, 1992, pages 7121-7127, XP002110936 the whole document</p> <p>-----</p>	1-15

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/GB 99/00874

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9853077	A	26-11-1998	AU 7501898 A	11-12-1998
US 5185440	A	09-02-1993	NONE	